HOMEOSTASIS OF CALCIUM

Discussion Leader:

DR. D. HAROLD COPP

COPP: The first point to consider in homeostasis of calcium is the remarkable constancy of the level of calcium in plasma and body fluids in the normal animal. This is illustrated in figure 1, which shows the

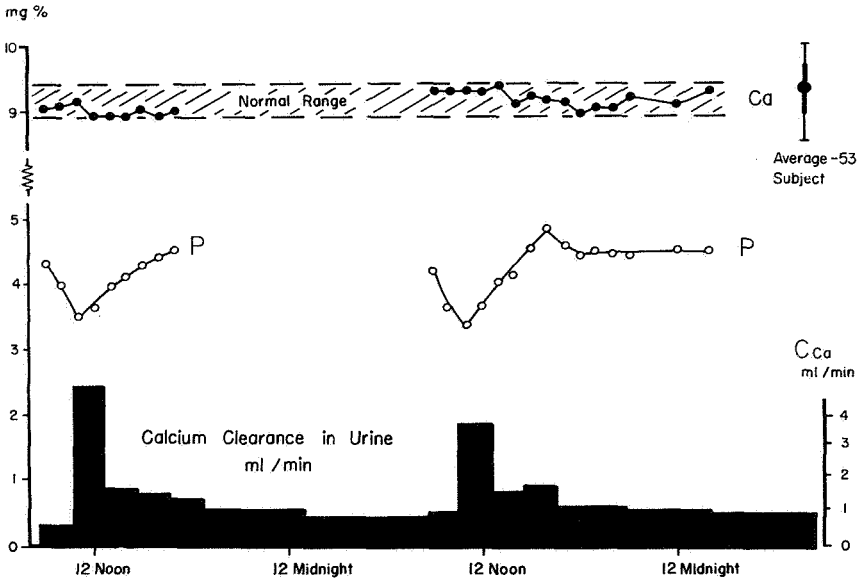


FIGURE 1. Diurnal fluctuations in plasma calcium and phosphorus levels in a normal adult male.

diurnal fluctuations of plasma calcium and phosphorus in a normal adult male subject in Shaughnessy Hospital, Vancouver, British Columbia. Although the calcium level is very constant, there is considerable fluctuation in the plasma phosphate and in the urinary excretion of both elements. The same constancy in plasma calcium has been observed in normal dogs and rats.

The main factors affecting plasma calcium are shown in figure 2 (ref. 11). These factors include absorption of calcium from food

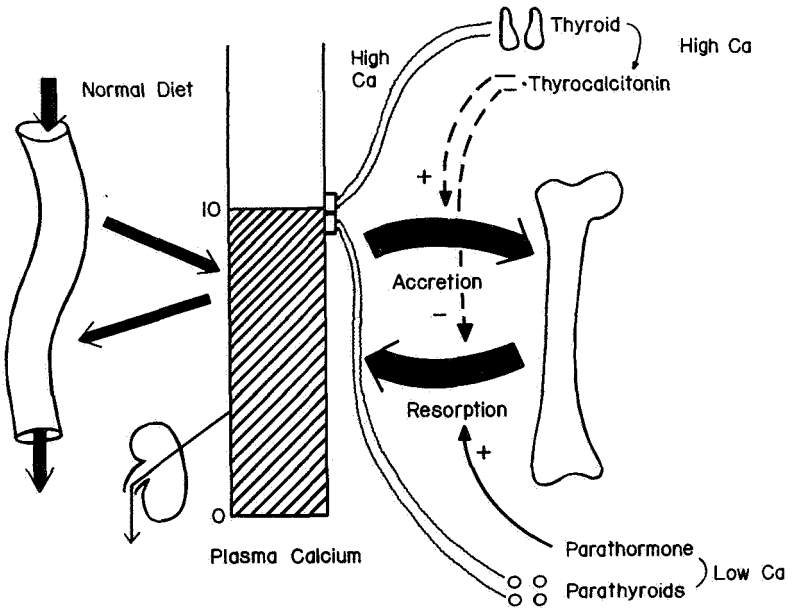


FIGURE 2. Factors involved in control of the plasma calcium level. [Adapted from ref. 11; reprinted by permission of the publisher.]

and excretion of calcium in feces, urine, and sweat. However, the most important single factor is the tremendous reservoir of calcium in bone, which may amount to over 1 kilogram in a 70-kilogram man. We believe that the tiny crystals of bone salt act like the particles of an ion exchange resin, providing a labile exchangeable calcium pool of 3 to 6 grams in a normal adult man, and that this acts as a buffer to help prevent wide fluctuations in the level of plasma calcium. However, in the long run, the balance between calcium accretion in bone formation and calcium release from bone during osteolysis is more important. I believe that these two processes are affected by the concentrations of calcium and phosphate in blood, by the presence or absence of vitamin D, and particularly by the activity of two potent hormones—the parathyroid hormone, produced by the parathyroids, and calcitonin, or thyrocalcitonin, from the thyroid. Diet is also a factor. Figure 3 shows that normal rats fed a phosphate-deficient diet are hypercalcemic in the morning.

RAISZ: Why do you think they are hypercalcemic in the morning?

COPP: Rats feed during the night, and increased metabolic activity requires phosphate for phosphorylative reactions. This increased activity, particularly on a phosphate-deficient diet, lowers the plasma phosphate, and in my opinion this result interferes with bone deposition of calcium. At the same time, calcium is absorbed from the gut at night.

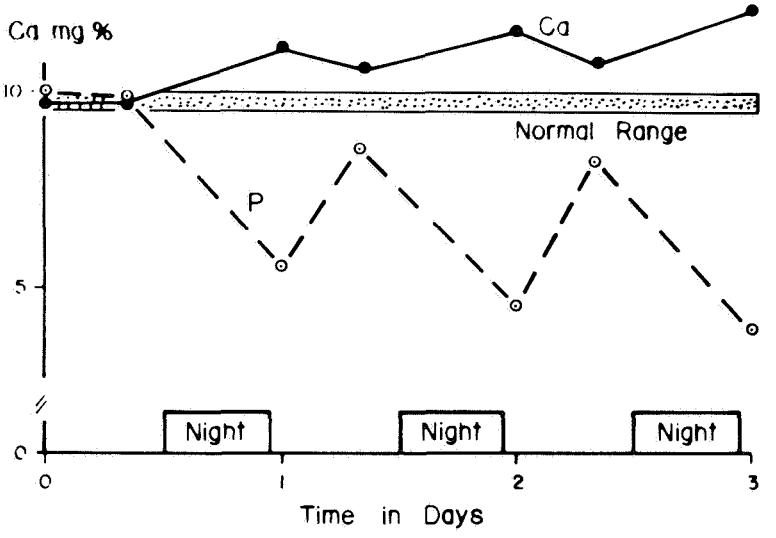


FIGURE 3. Changes in plasma calcium and phosphate in 5-week-old rats restricted to a diet low in calcium and phosphate. Note the marked changes in plasma phosphate associated with nocturnal feeding habits.

FREMONT-SMITH: Is most of the food intake at night?

COPP: Yes. Rats eat almost entirely at night. If you turn off the lights in the daytime you may be able to persuade them to eat something, but rats are rather shy and carry on most of their activities at night.

HOWELL: Do you have similar data for man?

COPP: No; we do not. It is almost impossible to get a phosphate-free diet that is acceptable to man.

NICHOLS: It is an important point then that this cannot be observed in man except to the degree that one can restrict the availability of dietary phosphate.

COPP: You may get some response in a hyperparathyroid patient with hypercalcemia when phosphate restriction may increase the plasma calcium level.

Our rats had essentially no phosphate in their diet and were growing rapidly, so that the phosphate required for soft tissues was obtained at the expense of the skeleton. We have shown that parathyroidectomized rats fed a low phosphate diet will develop hypercalcemia, even when the diet is also low in calcium.

PRITCHARD: I would like to ask a question in reference to what you have said. In reading about calcium and phosphate concentrations, one finds that one level always seems to go up as the other concentration goes down. Do calcium and phosphate always move in opposite directions?

COPP: Sometimes changes in calcium and phosphate move in the same direction, sometimes opposite. It depends on many factors.

PRITCHARD: I thought you were assuming that because the phosphate was down the calcium must go up.

COPP: No. With these phosphate-deficient diets we find that as blood phosphate falls, blood calcium rises. This is true particularly in the parathyroidectomized rat.

PRITCHARD: Is this a matter of mass action, or is it something biologic?

COPP: I think there may be two factors. Low concentrations of phosphate may affect the rate of bone deposition of calcium. At very low blood phosphate concentrations, there may be little or no formation of new bone mineral; high concentrations may have the opposite effect. This would account for a reciprocal relationship between calcium and phosphate and would account for some of the observations of Fuller Albright. I feel that an effect on bone formation would be critical. Phosphate deficiency also affects the response of the animal to parathyroid hormone and thyrocalcitonin. There will also be an effect on calcium absorption from the gut; this absorption is enhanced at low phosphate concentrations and reduced when the dietary phosphate is high. We observed that phosphate deficiency with low plasma phosphate also results in tremendous urinary excretion of calcium (ref. 12).

PRITCHARD: It is doubtless not a simple relationship.

COPP: That is true.

BAUER: In general, Dr. Copp, do your data show that your animals had enough vitamin D?

COPP: Yes. The animals received adequate but not excessive vitamin D. The diet contained 20 U.S.P. units vitamin D per gram, which is 7 times the recommended minimum.

I will assume that we all agree that plasma calcium is controlled with remarkable precision and efficiency in the normal animal. Figure 4 (ref. 13) shows that this precise control is lost when the thyroid and parathyroid glands are removed. In 200 normal dogs, the fasting plasma calcium concentration falls within a rather narrow distribution curve. In the thyroparathyroidectomized dogs, there is a great variability in the fasting plasma calcium concentration. This impairment in homeostatic control is demonstrated even more dramatically when the system is stressed by infusion of calcium or of the calcium chelating agent, EDTA (ethylenediaminetetraacetate). Sanderson et al. (ref. 14) found, in normal dogs (fig. 5), that the plasma calcium returned to the original concentration a few hours after it had been raised by infusion of calcium or lowered by injection of EDTA. This precise control was lost after thyroparathyroidectomy, as shown in figure 6, and the blood calcium was still not back to the original concentration

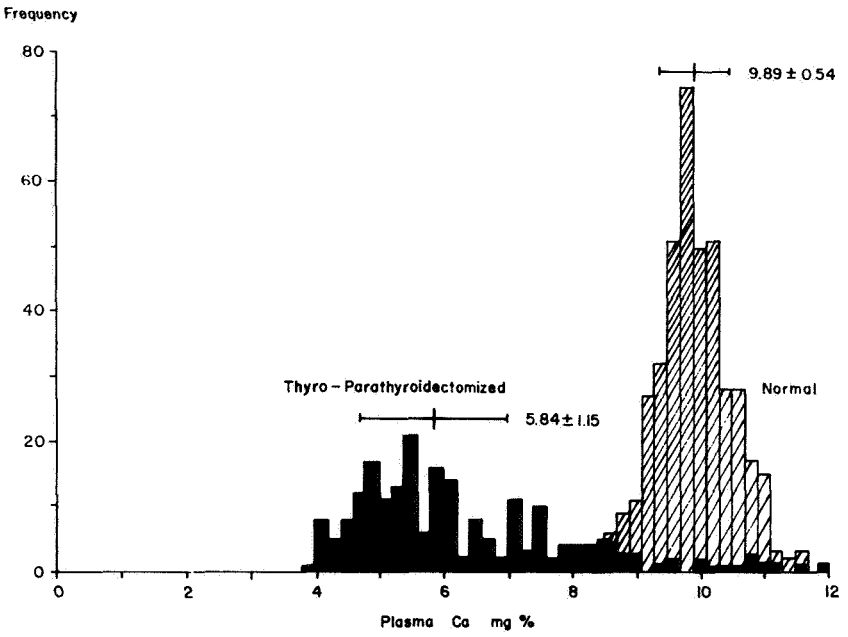


FIGURE 4. Distribution of fasting plasma calcium concentrations in normal and thyroparathyroidectomized adult dogs. [Adapted from ref. 13; reprinted by permission of the publisher.]

24 hours later. It is apparent that the glands were required for rapid, efficient homeostatic control of both hypocalcemia and hypercalcemia.

FREMONT-SMITH: There is a tendency to return toward normal values that starts quite promptly, is there not?

COPP: Yes. This tendency to return toward normal was observed even in the thyroparathyroidectomized dogs, but it occurred much more slowly.

FREMONT-SMITH: The reason I raise this point is because L. J. Henderson found, in his work on the constancy of the pH in the blood (ref. 15), that as one homeostatic factor after another was removed, secondary factors came in. I wonder whether there were not secondary factors to manage the calcium concentration in this situation.

COPP: Yes, I think so. There is a gradual recovery to the initial concentration within 24 to 48 hours following induced hypocalcemia or hypercalcemia. I believe that this is a result of a secondary and less sensitive control. If we assume that the rate of calcium deposition in bone is a function of the ionic calcium concentration, hypercalcemia should increase calcium deposition in bone and lower plasma calcium; yet hypocalcemia will have the opposite effect.

FREMONT-SMITH: Is the bone reservoir the main secondary factor?

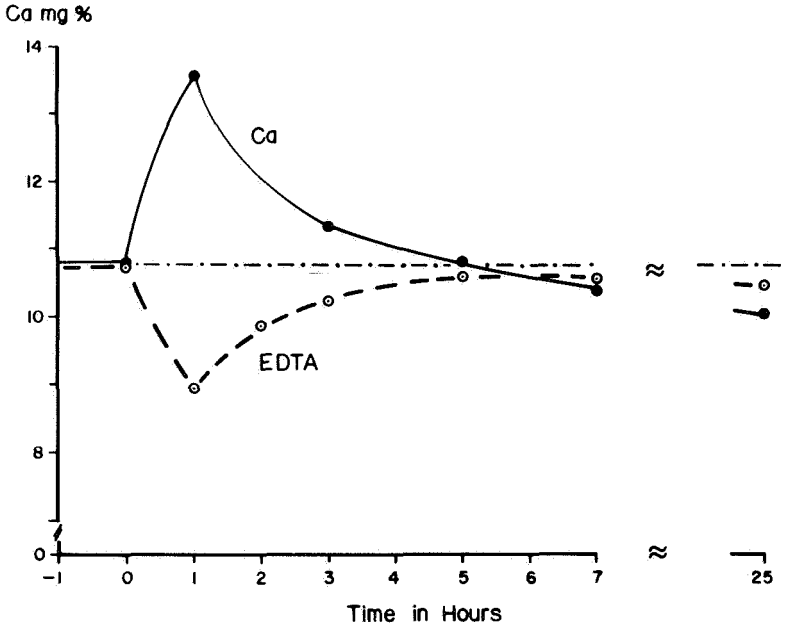


FIGURE 5. Changes in plasma calcium during and after intravenous infusion of calcium (15 mg/kg) or the EDTA equivalent of calcium (5 mg/kg) for 1 hour in normal adult dogs. [Replotted from data of Sanderson et al., ref. 14.]

COPP: Yes, and the relative balance between accretion and resorption.

PECK: What is the effect of age on this process?

COPP: Recovery is much faster in young animals than in old ones, not only with regard to the hormone response in intact animals, but also with regard to recovery in parathyroidectomized dogs.

HOWELL: In the dog, is there not a problem of accessory gland remaining in the mediastinum?

COPP: We do not worry about this. Our animals behave as if they were parathyroidectomized, and in over 200 operations we have seen no evidence of functional accessory glands.

TALMAGE: I would like to stress briefly the homeostatic control of calcium in the absence of the parathyroid gland. Even in the absence of the gland, plasma calcium concentrations tend to equilibrate, although at a lower concentration. However, because the equilibration process under these conditions is based primarily on physicochemical properties, the rate of equilibration is much slower. On a calcium-free diet, a basic level will be maintained and will be affected only by the low rate of calcium reabsorption by the kidney tubule. If an animal is maintained on a high calcium diet, the basic level may never be reached; the calcium concentration in the blood may be extremely

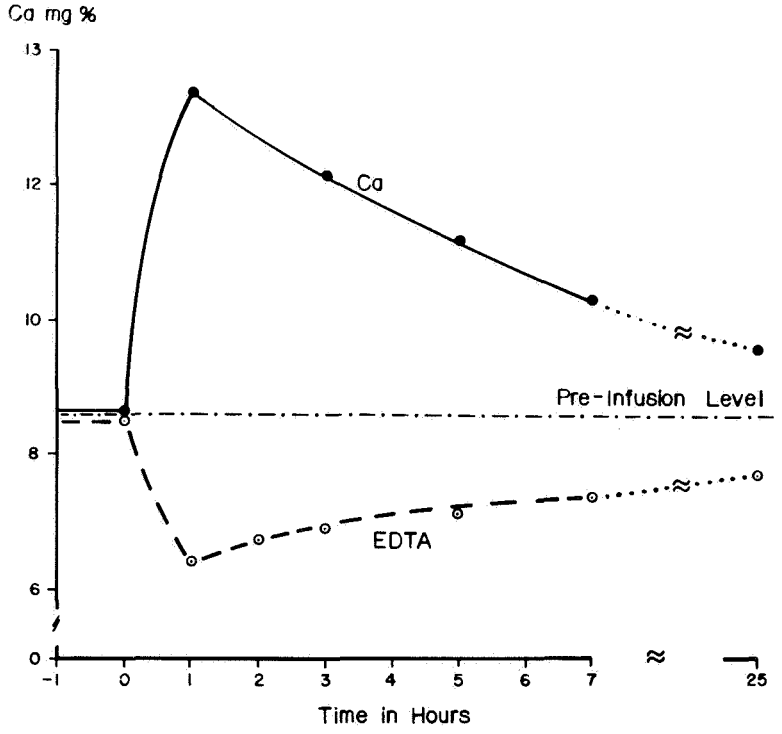


FIGURE 6. Changes in plasma calcium during and after intravenous infusion of calcium (15 mg/kg) or the EDTA equivalent of calcium (5 mg/kg) into thyroparathyroidectomized dogs. Note the impaired homeostatic control of both hypocalcemia and hypercalcemia. [Replotted from data of Sanderson et al., ref. 14.]

variable and may even reach a concentration higher than that seen in the normal animal. The important point is that even in a parathyroidectomized animal the bone can provide calcium to the fluid compartments.

COPP: This is one of the secondary factors that Dr. Fremont-Smith mentioned.

WALSER: I would like to raise a slightly semantic objection which I raised some years ago. EDTA does not induce hypocalcemia; it induces hypercalcemia. The same is true of citrate or protein. You can raise plasma calcium by infusing either. I do not think you would use the word *hypo*calcemia for those situations and I do not see why we should for this situation.

COPP: Your semantic point is well taken, but it does not alter the fact that EDTA produces physiologic hypocalcemia.

WALSER: I think the assumption that physiologic calcium has been measured by what you have illustrated is a little risky.

COPP: I do not agree. This is evident from the response of the parathyroids to EDTA-induced hypocalcemia.

RAISZ: I would like to carry this further. A question pertinent to your study is whether there is any effect of EDTA separate from the chelating of calcium. I agree that ionized calcium effects probably determine the responses in the whole animal where a modest amount of EDTA is being infused. When larger amounts of EDTA are given, however, we are no longer studying the effects on calcium but studying the effects of EDTA on tissue which can be substantial. EDTA has toxic effects on transport systems of cell membranes in many tissues that we have studied; these effects are entirely separate from the calcium effect.

COPP: I agree completely. EDTA complexes a number of important trace metals and prolonged administration may have deleterious effects. However, I do not think that these effects are important if you infuse EDTA for only a few hours, as has often been done in human subjects. But, for example, after 2 to 3 days of EDTA infusion in sheep, the animals often sickened and died.

TALMAGE: One must keep in mind that the same type of response may be obtained without EDTA. This response can be found in any method by which plasma calcium is lowered dramatically. The calcium invariably returns rapidly to the predetermined concentration.

NICHOLS: I do not think that EDTA's effect on tissues should be discussed as necessarily being unrelated to calcium. Indeed, it is probable that the transport effects you observe are actually effects on the membrane structure, which in turn are probably related to the binding of calcium into these membranes. For example, one can abolish sodium and potassium transport in red cells with EDTA and restore it again by adding enough calcium to saturate the EDTA present and leave a small excess (G. Nichols, unpublished observations).

RAISZ: But you can show that EDTA is bound to the membrane itself; that is, that even after all the calcium has been chelated there is still further binding of EDTA. Another point is that such ions as citrate, oxalate, or phosphate can be used to lower the calcium. We have had better results with these than with EDTA in the whole animal (ref. 16).

NICHOLS: I would like to comment about man and EDTA infusions. One can obtain data in man, which are not dissimilar from yours, Dr. Copp, as probably many here have done. Our own experience with man has been a fair copy of what you have shown in animals, with the exception that the duration of the whole phenomenon seems to be quite a bit longer. This is probably partly because we have infused for 4 hours rather than for 1 hour (with the specific intent of being sure the parathyroids have been shut off), but we do not get calcium returning to normal following EDTA infusion for quite a few hours. Incidentally, the same is true for calcium infusions in man.

COPP: This probably reflects differences in the metabolic activity of the skeleton.

ARNAUD: Dr. Nichols, are these data from a parathyroidectomized subject?

NICHOLS: No. The data were from normal people. If you have a parathyroidectomized subject the response is even slower. Another interesting phenomenon is that in carcinoma of the parathyroid, or occasionally in adenoma with hyperparathyroidism, serum calcium concentrations may go up and stay up after calcium infusions. We have seen them still elevated 4 days later.

COPP: The next two figures illustrate this point. Figure 7 shows the plasma calcium curve for a normal adult human male subject following a 1-hour calcium infusion. Recovery is somewhat slower than in the dog, but after 24 hours plasma calcium concentration is almost back to normal. Figure 8 shows the curve following a similar infusion in a thyroparathyroidectomized adult human male subject of similar age, and confirms Dr. Nichols' observation that the calcium concentration in plasma remains elevated.

NICHOLS: Dr. Talmage points out that this is probably related to the fact than an adult man does not use his gut as a calcium pool for

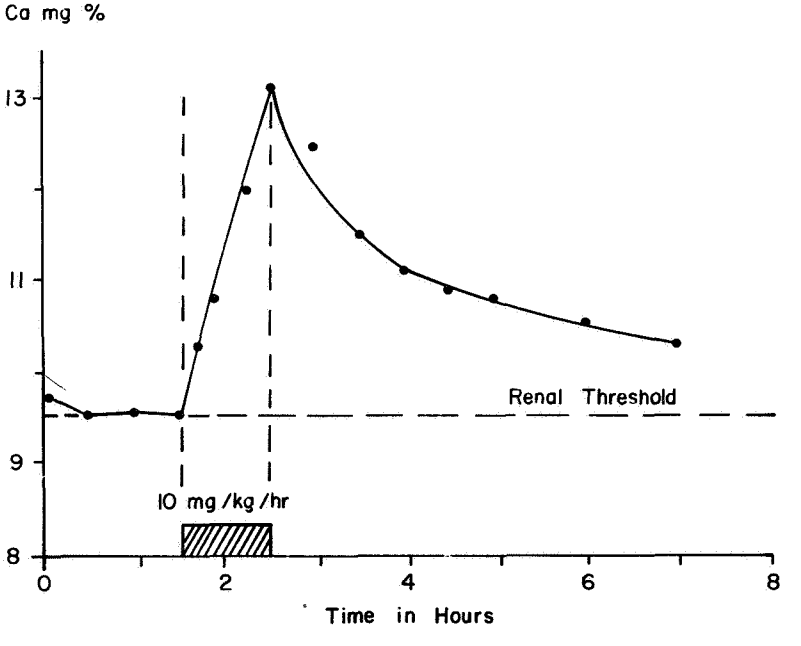


FIGURE 7. Changes in plasma calcium during and after intravenous infusion of calcium (10 mg/kg) in a normal adult man. [From the Clinical Investigation Unit, Shaughnessy Hospital, Vancouver, B.C.]

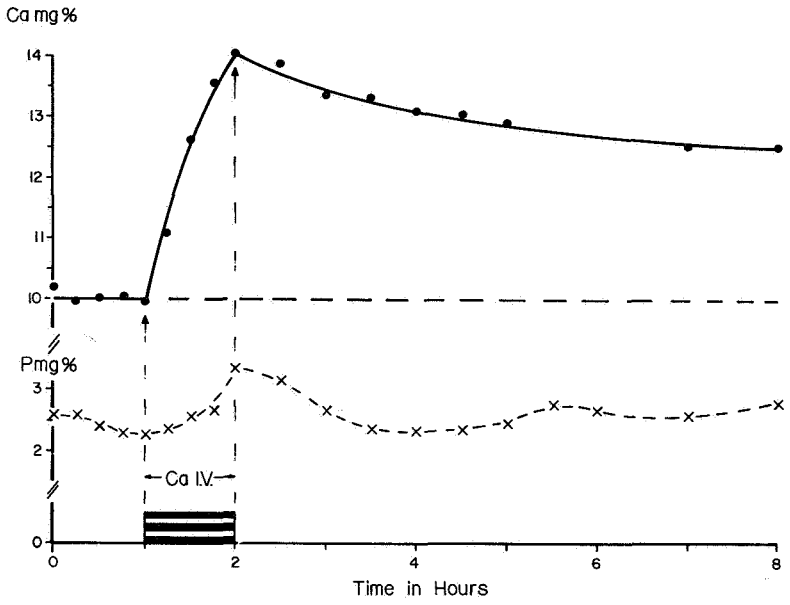


FIGURE 8. Changes in plasma calcium during and after intravenous infusion of calcium (10 mg/kg) into an adult man who had been thyroparathyroidectomized a number of years earlier. Note the impaired control of hypercalcemia. [From data supplied by Dr. H. W. McIntosh, Department of Medicine, University of British Columbia, Vancouver, B.C.]

ready availability but instead depends upon his bone which is kept in a state of availability by the presence of parathyroid hormone.

HOLTZER: How did this patient have a normal calcium concentration in the beginning? Was he on vitamin D therapy?

COPP: He was maintained on a high calcium diet of 0.2 milligram thyroxine and 50 000 IU vitamin D daily.

The second item to be considered in our discussion should be the function of bone in calcium homeostasis. Over 99 percent of the calcium in the body is located in the skeleton, and this amount may be over 1 kilogram calcium in a 70-kilogram man, as mentioned previously. It is also probable that the regulating hormones from the thyroid and the parathyroid glands exert their effect primarily by action on bone. Considering the nature of bone mineral, we find the most significant physical feature is the extremely minute dimensions of the crystals, which provide an enormous area for surface exchange. In many respects these crystals are comparable to the resin particles of an ion exchange column and act in much the same way to release or to adsorb ions present in the surrounding body fluids. Figure 9 shows a stylized model of such a crystal and indicates the way in which calcium on its

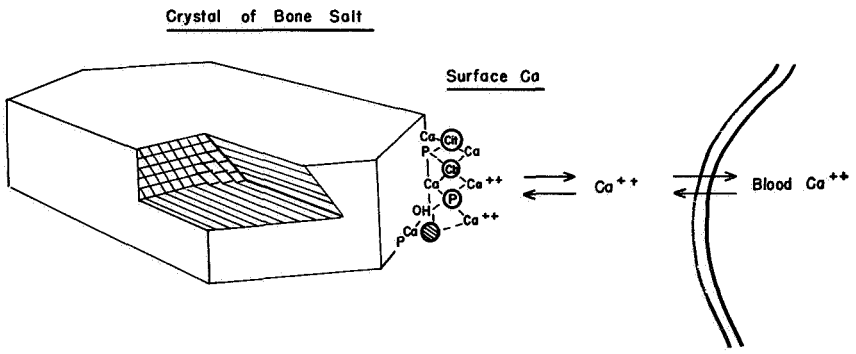


FIGURE 9. Diagrammatic model of exchange between calcium in blood and calcium on the surface of the crystals of bone mineral.

surface could exchange with calcium in plasma. In my opinion, this surface exchange is a physicochemical process that is independent of the energy-dependent and biologically active processes of accretion and bone resorption.

Dr. Robinson, will you comment on this and on the ultrastructure of bone where these processes probably take place?

ROBINSON: The crystal in figure 9 is one which I think is perfectly valid. With better resolution we still observe that those bone crystals large enough for their habit to be observed are tablet shaped. According to X-ray and spot electron diffraction patterns their fundamental atomic lattice pattern is that of hydroxyapatite with some variability of their simple surface composition. These small crystals are probably flattened plaques, which increases the surface-to-mass ratio; these crystals are supposed to occupy, as we conceive the process in mineralization, water space that originally existed in the matrix shortly after this matrix was formed by the cell.

As the crystals occupy this space and the water is displaced to give them room, less and less water becomes available for ion exchange in areas around the surface of these crystals. Finally, the situation is such that in a fully mineralized bone matrix, the crystals on the periphery of the mineralized matrix masses are the only ones in actual continuity with extracellular fluids free enough to allow ion transfer.

PRITCHARD: Dr. Robinson, how big are those mineralized matrix masses? What should be one's picture of a mineralized mass of crystals, and are they really crystals?

ROBINSON: Yes; I believe they are crystals.

PRITCHARD: Is that mass the whole mass between one blood vessel and the next?

ROBINSON: No; this is extremely variable. In some areas in bone the matrix is very poorly mineralized and in other areas the matrix is rather compact. I will discuss this subject in more detail later.

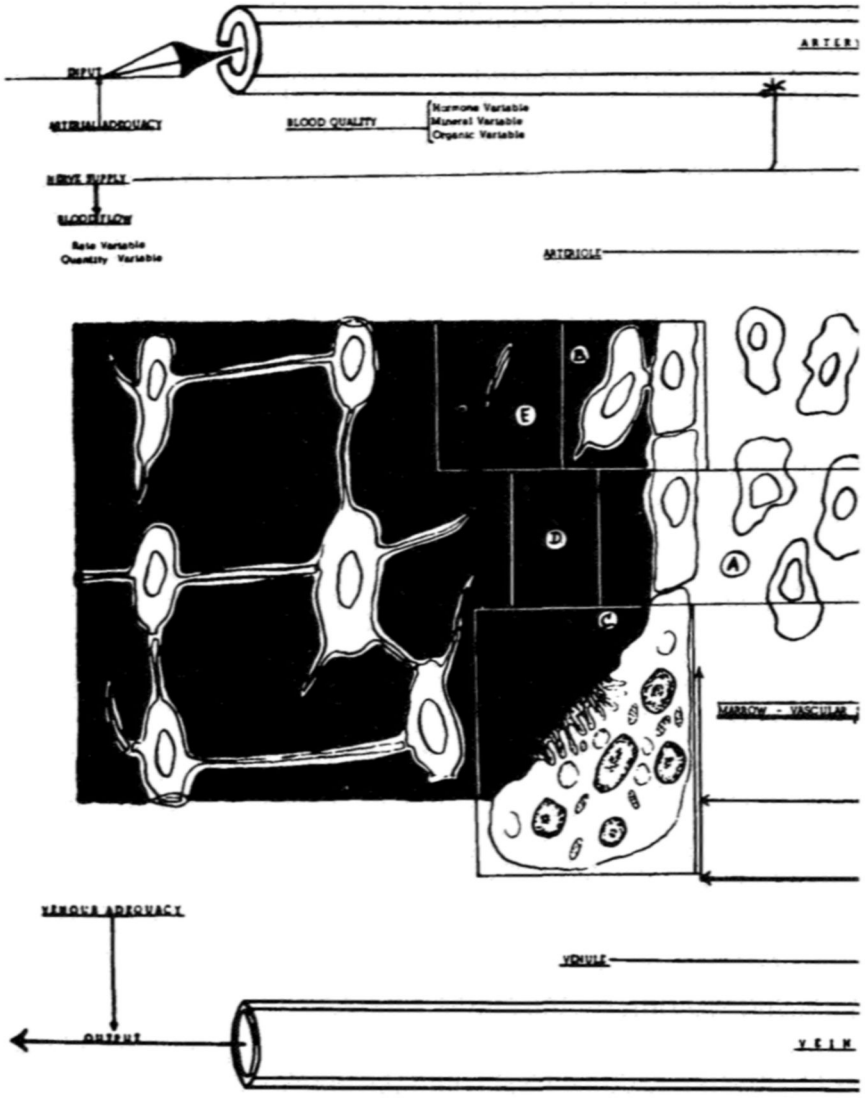
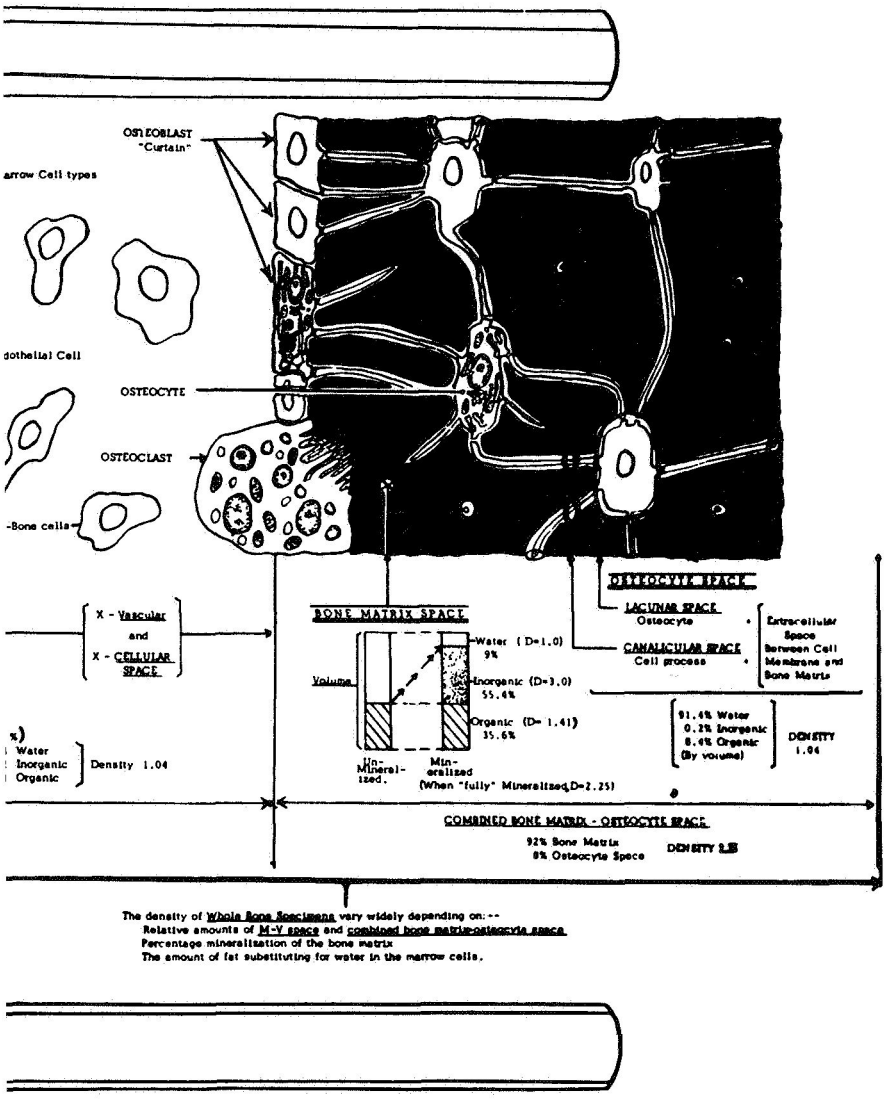


FIGURE 10. Bone as a physiologic unit. [From re

Figure 10 is a diagram illustrating the basic concept of the physiologic unit of bone (refs. 17 and 18). This concept shows the influx of blood and the outflow of blood; there are endothelial cells with a basement membrane which, incidentally, forms the container for the blood as it goes through the bone. Between the surfaces of these endothelial cells and the mineralized matrix, one can occasionally see



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polymorphonuclear cells and a variable number of other cells. (The electron-microscope studies reported here were supported by Public Health Service, National Institutes of Health (NIH) research grant no. AM00706 and NIH training grant in orthopedic surgery no. TI AM 5317.)

In some places, particularly on the surface of newly forming bone

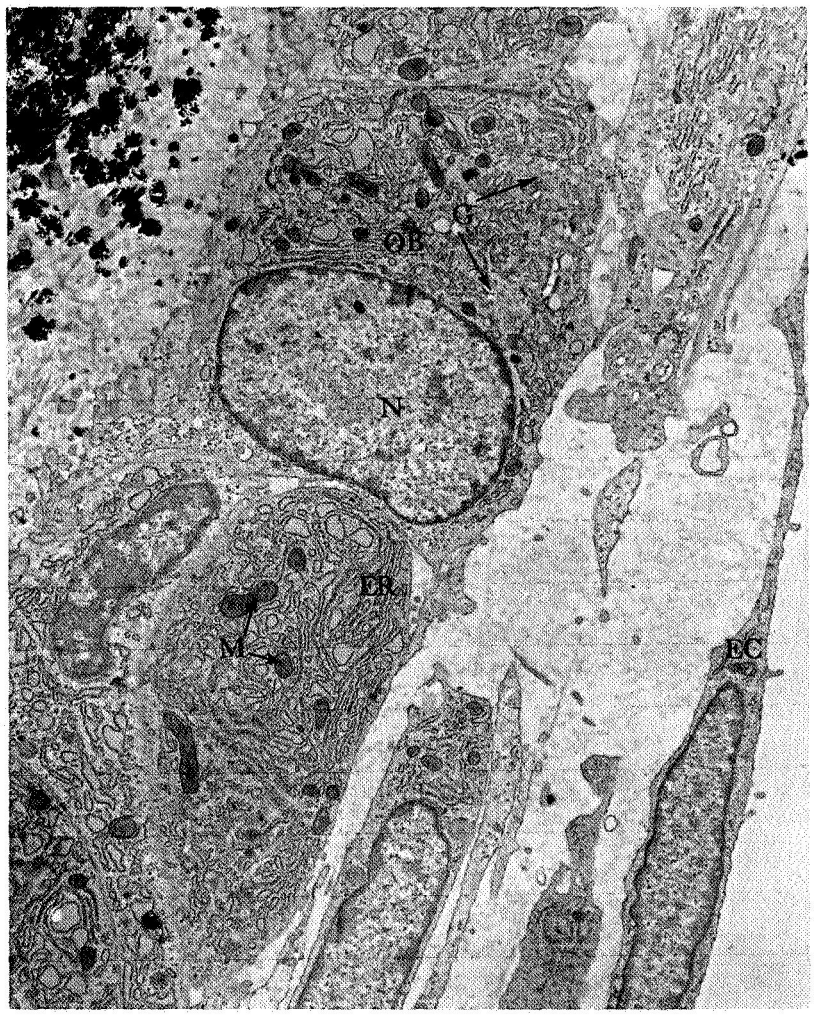

FIGURE 11. An electron micrograph of the surface of metaphyseal trabecular bone from a young growing rat. On the far right lower two-thirds of the figure is a vascular lumen. Then, proceeding to the left upper corner, one observes an endothelial cell (*EC*), several intermediate cells among which are probably some preosteoblasts, and then a layer of closely packed osteoblasts (*OB*). The osteoblast cytoplasm contains a well-developed rough endoplasmic system of membranes (*ER*), mitochondria (*M*), a juxtanuclear Golgi apparatus (*G*), and a nucleus (*N*). The osteoblasts in this situation appear to form a curtain over the underlying bone matrix. In the left upper corner, one observes the very incompletely (spottily) mineralized collagenous bone matrix. However, as one can observe in figure 12, the mineralization becomes quite complete in this type of specimen within a few hundred angstroms of the collagenous periphery of the bone-matrix mass. Approximately 6780×. [Reprinted by permission of Dr. David Cameron, University of Sydney, Sydney, Australia.]

on metaphyseal trabeculae (figs. 11 and 12) and along the walls of vascular canals in osteons that are actively filling (fig. 13), osteoblasts form a closely packed, continuous layer of cells over the bone matrix. However, in regions of longer standing bone matrix, the osteoblasts,

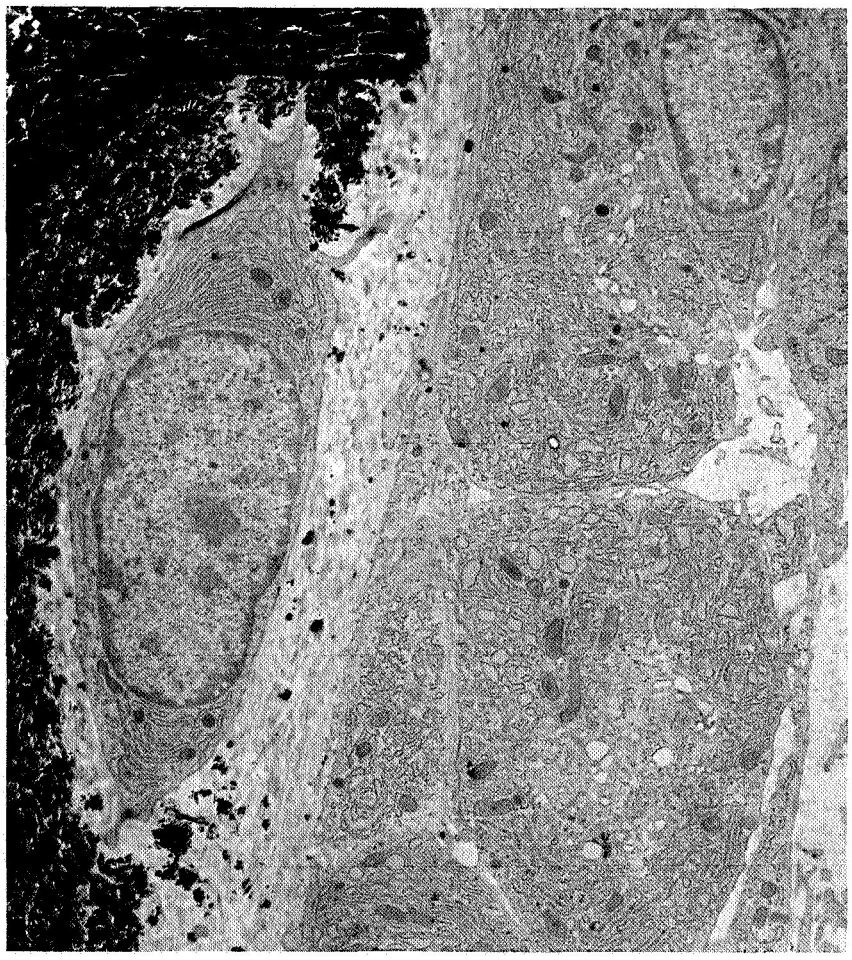

FIGURE 12. The topography and magnification of this figure is similar to that of figure 11. However, in this micrograph one can observe an osteoblast becoming surrounded by bone matrix. As noted in the text, there are some round, dense masses of detritus overlying the cells and matrix which are probably small contaminating deposits of lead acetate. On close inspection these can be distinguished from the rough clusters of bone crystals forming spottily in the generally unmineralized bone matrix surrounding the surface osteocyte and underlying the curtain of four or five osteoblasts. The more fully mineralized bone matrix can be seen in the left upper corner about 1 micron from the nucleus of the osteocyte. Approximately 6780 ×. [Reprinted by permission of Dr. David Cameron, University of Sydney, Sydney, Australia.]

or bone-matrix-covering cells, may be strung out in a tenuous cell cover over this matrix. In electron micrographs of haversian canals in midfemoral cortex of adult dogs, there appear to be spaces between or fenestrations through these bone-matrix-covering cells; one would think these spaces might allow unobstructed contact of extracellu-

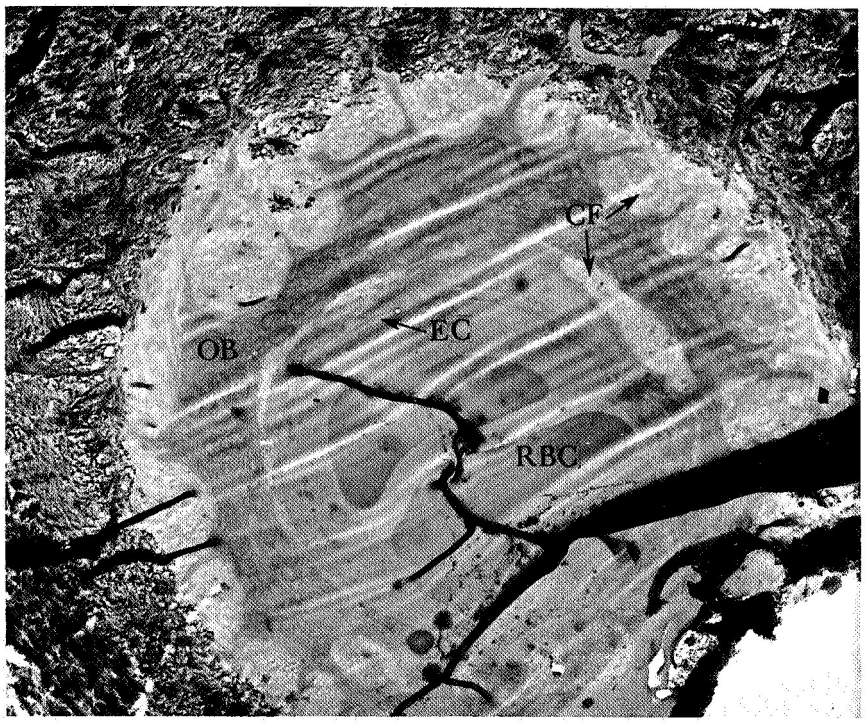

FIGURE 13. Electron micrograph of a cross section of a haversian canal from the mid-diaphyseal cortex of the femur of a puppy. The dark black lines and white streaks are wrinkles in the section. Near the center are two red blood cells (*RBC*) in the lumen of the haversian capillary. The capillary is usually surrounded by two or three endothelial cells (*EC*). They have a basement membrane. The intermediate cell (*IC*) is not further described because it could be a preosteoblast, a pericyte, or an extension of an overlapping endothelial cell. Particularly in canals in which osteoblasts (*OB*) appear to be in an active phase of protein synthesis as do those in this figure, one or more of these intermediate cells are seen.

Collagen fibrils (*CF*) in this specimen (which was fixed in glutaraldehyde, embedded in Epon, and counterstained with a lead acetate solution) appear as white disks in a more dense (darker staining) cement substance. Also note that unlike the situation at the periphery of trabecular bone, bundles of collagen fibrils appear between the vascular space and the osteoblast curtain; occasionally mineralization is observed in these regions as well as in the bone matrix placed centrifugally to the osteoblasts. Approximately 3570×. [Reprinted by permission of Dr. Reginald Cooper, University of Iowa Medical School.]

lar fluid with bone matrix. Therefore, the cell cover is morphologically different over the surface of forming and newly formed bone matrix and over the surface of longer standing, quiescent, or resting bone where there is no morphologic evidence of bone resorption or accretion. Of course, *in vivo,* this tenuous cell cover over the surface of a mass of completed and mineralized bone matrix may be a dynamic one with more power over the physical state of the extracellular fluid, which "laps up" on the "shore" of mineralized bone matrix masses, than is suggested by morphologic observations such as these. Such resting osteoblasts may respond in an as yet undelineated manner to the influence of hormones, vitamins, nerves, and chemical and pH changes in their environment.

The ratio of the mass of mineralized bone matrix to the available crystal surfaces on its periphery is difficult to calculate for two reasons: first, the crystals project irregularly into extracellular and free bone matrix fluid along the periphery of a mass of mineralized bone matrix; second, the periphery of even a fully mineralized bone matrix mass may be, for a depth of several hundred angstroms, incompletely mineralized. Therefore, one cannot exactly calculate the surface-to-mass ratio as one could if the surfaces of the mineralized bone matrix masses were smooth and if full mineralization of their bone matrix extended right up to those smooth surfaces. In other words, there is a surface roughness factor and a peripheral depth factor which make surface-to-mass calculations difficult.

I attempted to estimate the apatite crystal surface area which presents on the periphery of the mineralized bone matrix masses in the cortical-type regions of the skeleton in a 70-kilogram man. I concluded that there should be between 1500 and 5000 square meters of bone crystal surface exposed to extracellular fluid on canalicular, lacunar, and vascular (haversian) canal surfaces. The variable factors of roughness and peripheral ion permeability of bone matrix masses tend to make this estimate of lesser magnitude than the true value (ref. 18).

PRITCHARD: I was actually referring to the little units bounded by the canalicules. Presumably these have a volume of the order of a cubic micron and a calculable external surface area. But then, there are the surfaces of the individual crystals inside this mass, and one does not know what pathways exist between these surfaces and the major pathways that you have shown.

ROBINSON: That is correct in terms of direct observation. However, on the basis of many experimental data (refs. 19 and 20), I finally developed a theory (ref. 17). I have chosen to call this the water-bridge theory. It explains the major factor governing the transit of calcium ions to and from the surfaces of individual apatite crystals

in the mineralized bone matrix masses of the skeleton (ref. 21).

The water-bridge theory applies only directly to those ions or molecules that require a certain freedom of water molecules for transit through extravascular and extracellular fluid. For instance, this theory does not apply to noble gases; radon apparently does not need water molecules to move into and out of mineralized bone matrix. In relation to bone, the water-bridge theory is as follows:

1. Bone matrix is formed by osteoblasts. When first synthesized prior to matrix mineralization, the matrix is highly hydrated and does not contain bone crystals. It does, however, per unit volume, contain its full complement of organic solids (ref. 22).

2. As bone matrix mineralizes, the bone crystals displace mainly water, volume for volume, rather than the collagenous organic solids which form the bulk of the organic solids in bone matrix. In cartilage (ref. 23) and possibly in bone (ref. 24) a large part of the noncollagenous protein, associated with the mucopolysaccharide of the original organic matrix, and some sulfated mucopolysaccharide are apparently displaced from any given volume of matrix, during mineralization. Some free fibrous (collagenous) protein molecules that are not incorporated or adequately crosslinked into fibrils may move out of the matrix prior to and during mineralization. However, in normal bone, the collagen solids, per unit volume of bone matrix, are not lost during matrix mineralization.

3. The original volume of bone matrix does not shrink or expand during mineralization.

4. Bone matrix water is not completely displaced during mineralization. At full mineralization, the residual bone matrix water becomes bound and can no longer form a water bridge for the transport of calcium ions. Each calcium ion needs, theoretically, two water molecules for rapid movement. These water molecules must have a degree of freedom not present in residual bone matrix water in fully mineralized bone matrix. This, of course, does not prevent the transit of calcium ions to the surfaces of bone matrix mineralization. It is our hypothesis, for instance, that diffuse exchange, at the light-microscope level of bone organization, in terms of ^{45}Ca autoradiographs, is actually caused by beta particles emanating from ions of the calcium isotope which have found or are finding their way via extracellular fluid to crystal surfaces on the walls of canaliculi which permeate the fully mineralized bone matrix. The walls of canaliculi are normally fully mineralized right up to the extracellular fluid-mineralized matrix mass interface. Proof or rejection of this hypothesis is being pursued at present by the use of combined electron micrography and autoradiography (ref. 21).

Part 4 of this theory applies generally to availability of mineral

crystal surfaces or mineral agglomerates and mineral-organic agglomerates other than crystalline, in any organic matrix, and not only in bone, but also in cartilage, and in ectopic sites of ossification and calcification. If there is no free water bridge in a tissue, then the ions and molecules that depend on this bridge for transit cannot move through the tissue to preferred sites of fixation or exchange.

5. Implicit in this theory are the following two corollaries:

a. The density of electrostatic forces progressively increases per unit volume of bone matrix as mineralization increases, limiting the movement through the matrix of particles carrying charges.

b. Surface-adhering forces that are of lesser strength than electrostatic forces and that are active even on particles not carrying a charge will increase per unit volume of bone matrix as crystal and fibrous protein surfaces become crystals on the periphery of a mass of fully mineralized bone matrix.

Conversely, when bone matrix mineralization is quite incomplete, e.g., when it contains one-half or one-tenth as much bone mineral per unit volume as it can contain when fully mineralized, then a water bridge of a sufficient number of free water molecules is present for the rapid transit of calcium ions to the surfaces of most of the bone crystals in the whole mass of quite incompletely mineralized bone matrix. If this volume of quite incompletely mineralized matrix is considerable in any one region of the skeleton, a hotspot (in terms of autoradiographs viewed at the light-microscope level of tissue organization) is present, and a region of low microradiographic density is present.

In the case of the periphery of fully mineralized bone matrix masses, the surface consists of bone crystals and possibly some noncrystalline mineral agglomerates, the surfaces of which are in continuity with extracellular fluid. The water molecules of the extracellular fluid have sufficient freedom to act as a rapid transport device for those ions and molecules which need free water molecules for movement.

The periphery of a fully mineralized mass of bone matrix may be more or less incompletely mineralized for a varying depth. The amount of calcium isotope picked up on the periphery of an otherwise fully mineralized matrix mass will be dependent on the depth of the peripheral zone of incomplete mineralization and is progressively more closely approximated as full mineralization is approached.

These forces—the electrostatic and surface-adhering forces—are conceived of as halting the rapid movement of all particles, including residual matrix water molecules, as bone matrix achieves full mineralization.

The concept of the mineralized mass is best shown in relation to

some work by Cooper et al. (ref. 25) on the haversian canals and their structure. The cortical or compact bone of the long bones of man, for example, is a major contributor to the total bone mass in the whole body. Because there are so many square meters of crystal surface on the periphery of fully mineralized bone matrix masses in cortical bone which face on intracortical vascular canals, canaliculi, and lacunae, one hesitates to regard this vast crystal surface-extracellular fluid interface as an unimportant part of the mineral homeostatic mechanism. Osteoblasts and osteoclasts are in close proximity to this interface and are certainly implicated in everything that takes place there.

I am concerned that metaphyseal bone, particularly that portion lying on the periphery of metaphyseal trabeculae, is usually considered to play the predominant role in mineral metabolism, when the cortical bone forms a much greater portion of the total bone mass in the skeleton and presents an extensive area of bone crystal surface to extracellular fluid in close association with bone cells. These morphologic factors, as I see it, make cortical-type bone more important than trabecular-type bone in total mineral homeostasis.

Figures 13 to 16 show electron micrographs of intracortical vascular (haversian) canals, canaliculi, and lacunae obtained from the midcortex of the femoral diaphysis of puppies and adult dogs.

Figure 13 is a cross section of an actively filling-in haversian canal from a puppy. One observes the blood vessel composed of two or three endothelial cells. Peripheral to this vessel along the bone matrix wall of the canal are several osteoblasts. One can observe the protoplasmic extensions from these osteoblasts extending through canaliculi into the bone matrix. These are particularly well seen in figure 14.

The little bone crystals are located in the region of mineralized bone matrix. Between the osteoblasts and the mineralized portion of the bone matrix is a zone of nonmineralized matrix. More peripherally, one observes mineralization of the matrix. This initial mineralization is spotty and is localized in and about collagen fibrils. A few hundred angstroms deeper into the bone matrix the mineralization is more complete, and crystals obliterate the images of collagen fibrils and the space between the fibrils. There are about 5000 to 10 000 angstroms of matrix space between the surface of the lining cells of this part of this haversian canal and the more fully mineralized portion of the bone matrix mass.

The peripheral zone of nonmineralized and partly mineralized bone matrix is highly hydrated. In the zone where mineralization of the bone matrix begins, the hydration starts to diminish and the crystal population starts to increase without any expansion or con-

traction of the original unmineralized volume of this matrix. As the mineralization becomes more and more complete, the residual matrix water would, according to the water bridge theory, be progressively more bound and decreased in amount per unit volume of

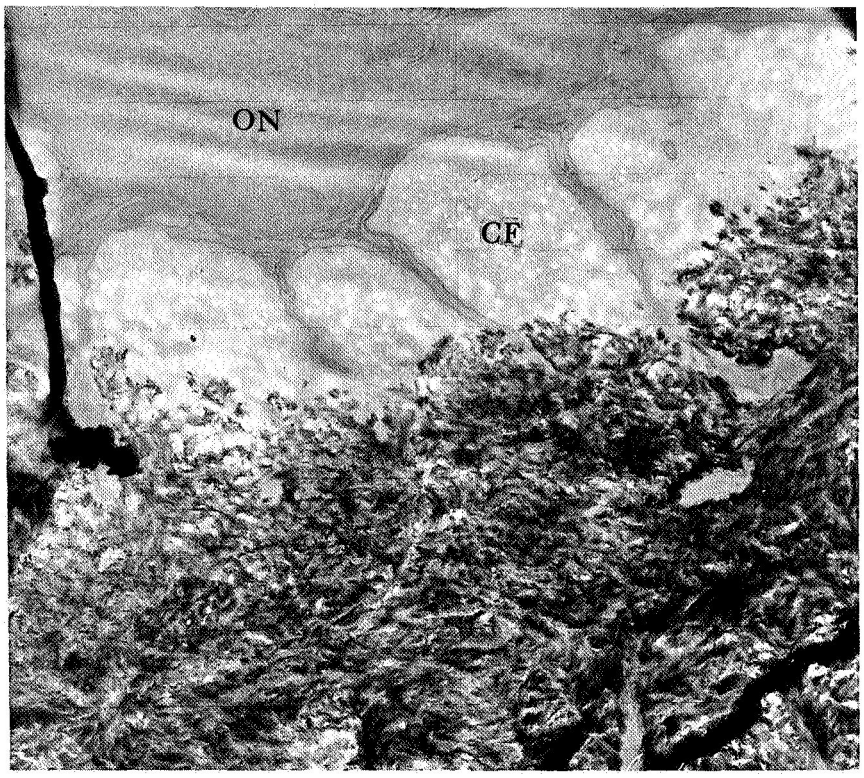

FIGURE 14. Electron micrograph in which the osteoblast-bone matrix interface at the periphery of a haversian canal is illustrated. At the top an osteoblast nucleus (*ON*) predominates the scene. A thin zone of osteoblast cytoplasm with fingerlike projections borders the nucleus. The cytoplasmic projections covered by plasma membrane are observed to extend through lacunae into the bone matrix. The bone matrix is unmineralized close to the osteoblast, and the collagen fibrils (*CF*) cut transverse to their long axes are seen as circular zones in a more densely staining ground substance which contains Epon and was counterstained with lead acetate. The mineralization of the bone matrix becomes more complete as one moves farther away from the cell. The roughness of the mineralized periphery of the calcified matrix mass, represented by bone crystal clusters, and the depth factor, represented by bone matrix in which incomplete mineralization is present on the periphery of the mineralized bone matrix mass, are both obvious in this picture. In regions of newly mineralizing matrix it would appear that (in this instance under very high magnification) the cement substance between the fibrils was mineralizing prior to fibril mineralization. Many exceedingly small crystals can be seen in this region. Approximately 18 000 ×.

bone matrix, and thereby prevent any rapid exchange of calcium ions between those ions on crystal surfaces in the more fully mineralized region and those ions in the extracellular fluid in the unmineralized matrix region at the periphery of the haversian canal.

There are a good many artifactual cracks in the mineralized matrix in our early specimens, but these cracks have been eliminated in many later sections of fully mineralized cortical bone by formulating the embedding medium used to infiltrate the haversian canals so that it equalizes any strain between itself and the surrounding fully mineralized bone matrix.

One can see that the surface of a mineralized bone matrix mass, in terms of bone crystal surfaces, is anything but smooth, and that the phase change from nonmineralized to fully mineralized bone matrix may be a gradual one giving depth to the periphery of the mineralized bone matrix mass.

BÉLANGER: Could I make a point here, Dr. Robinson? I think one thing that confuses people when they are talking at the same time of electron micrographs and microradiographs is that microradiographs are generally first image pictures, otherwise called a negative, whereas an electron micrograph is a photograph, or a print, so that what is dense, or black in your electron micrographs, is white in the microradiograph. I just want to clarify this point.

TALMAGE: Dr. Robinson, these figures show primarily osteoblasts bordering the canal. If these osteoblasts are actively synthesizing collagen, what prevents the center from filling in with matrix? How does it maintain its constant size?

ROBINSON: As an osteon is formed, a state is reached in which no more closure or encroachment on the vascular space in the center of the osteon occurs. Equilibrium is established.

TALMAGE: Are not the osteoblasts still synthesizing collagen?

ROBINSON: Figure 13 happens to be from a puppy. It has obviously reached a point where, if the peripheral osteoblasts synthesize much more bone matrix, they would shut off their own fuel supply. I think that some factor comes into play at this point which prevents this. I do not know what the factor is.

CURREY: Are three distinct layers usually found in a fully mature haversian system?

ROBINSON: Even about the periphery of a fully mature section of a haversian canal one usually can find three distinguishable zones along some segments of its periphery (fig. 127). In this regard, great care must be taken during the preparation of the material to avoid any demineralization. When these sections of bone are produced by a special microtome, the section is only about 500 angstroms thick. These small sections float out as a ribbon over a water bath, and then

they are picked up on a grid. If the pH falls below about 6.8, these sections will demineralize very rapidly. In this work it is essential, therefore, to keep the pH elevated.

Boothroyd (ref, 26) wrote a paper about this subject, and Dudley and Spiro (ref. 27) earlier pointed out the problem of demineralization on the surfaces of these matrix masses in such thin sections. Even with great care, one must always be suspicious that some demineralization could have occurred. However, I think we have this problem under control and do not feel that this area of unmineralized matrix is an artifact.

ARNAUD: How do you control the problem?

ROBINSON: We place an indicator in the fluid and we keep the pH at 7.2.

ARNAUD: What happens if it changes?

ROBINSON: We change our solution. As the color starts to change we change our solution.

BÉLANGER: Is there a possible loss at pH 7.2?

ROBINSON: We have not seen any, whereas the loss becomes very dramatic below pH 6.8.

OWEN: In figure 13 it seems as if there is a distinct boundary between the three zones, rather than a gradual merging of the zones. Is this correct?

SAXÉN: Would you indicate the cell surface, or the cell membrane?

ROBINSON: Figure 14 is a more highly magnified electron micrograph than figure 13, and shows more clearly the plasma membrane of the osteoblast in relation to the bone matrix. This figure shows the plasma membrane covering the protoplasmic extensions of the cell which proceed into the bone matrix.

The crystal masses start to form spottily through the mineralized zone. Nevertheless, there is a clear demarcation from the next zone, in which mineralization is fairly good, but definitely incomplete in some areas; then, in the next zone mineralization is suddenly quite complete.

BUDY: In figure 14, what are the vacuoles below, above, or in between the mineralized area?

ROBINSON: Those are collagen fibrils, and the space between the fibrils is more dense in this micrograph than the fibrils themselves. This may be because the embedding media picked up some of the lead counterstain, whereas the fibrils themselves did not pick up so much.

If the mineralization were all peripheral to the collagen fibrils, one would see holes the size of the collagen fibrils throughout the mineralized matrix mass when the mineralized matrix mass is sectioned transverse to the long axis of the collagen fibrils. We have not seen

these holes, and this is one of the major arguments for the belief that these mineral crystals occupy space in the fibrils, as well as around the fibrils in the mineralizing region.

PECK: You cannot exclude the possibility, though, that those spaces are occupied by lipid.

ROBINSON: The collagen fibrils are laid down around the osteoblasts that line the haversian canal. These osteoblasts are laying down the fibrils in one direction, but apparently every few hours they switch the collagen axis and lay them down more or less at right angles to the previous direction. The fibrils are usually more osmiophilic than the material in the regions between them. Therefore, I have no evidence that there is much lipid present.

MACDONALD: Dr. Robinson, would you say this is evidence that calcification not only occurs at the surface of the mineralization front, but also can occur at random in the matrix above it?

ROBINSON: That is correct. The mineralization front is not observed to be a straight line that moves from the fully mineralized matrix into the newly formed matrix. Rather, mineralization first appears spottily throughout the newly formed matrix. The cell produces the matrix; then there is a lag period. This lag period can be extended in several situations. One of the most clinically outstanding situations is the fracture callus, where a great deal of matrix mass may be formed before mineralization appears.

Cameron has mentioned this (ref. 28), particularly in the fracture callus of the rat.

NICHOLS: How does the mineral get there, by diffusion?

ROBINSON: Right. First, the matrix mass does not decrease or increase in volume during mineralization, and second, the density increases rather suddenly in those particular regions where mineralization is occurring. This increase implies diffusion of mineral ions into crystal nucleation sites at the time of mineralization and not significantly before. On the basis of electron micrographs of mineralizing matrices of epiphyseal cartilage and of bone, as previously noted (refs. 21 and 29), an amorphous "cloud" more dense than the surrounding ground substance seemed to appear about a portion of one collagen fibril or about adjacent portions of several fibrils. It was in this cloud and in relation to a collagen fibril that the first crystals seemed to appear. The most logical explanation is that a chemical change occurs in a portion of the newly formed bone matrix. This change involves the collagen and a surrounding portion of ground substance. The altered portion of the bone matrix then becomes a "trap" for calcium and phosphate ions. Diffusion into this trap is possible because the water bridge for calcium ions is open between the matrix trap and the vascular space.

In 1963 I speculated that an enzyme produced by a cell might, as suggested by Campo (ref. 23), split the noncollagenous protein from the mucopolysaccharide, permitting chondroitin sulfate to act as a calcium trap while the adjacent collagen fibril became a phosphate trap (ref. 30).

If the calcium ions were already in the matrix and if only the water was removed at the time of mineralization, a vacuum would be formed or the matrix would decrease in volume. Furthermore, if calcium ions were already in newly formed bone matrix at a density even approaching the density they assume during apatite crystal formation, microradiographs of freeze-dried bone and microchemical analyses should show their presence, but they do not (ref. 24).

It appears to me that the mineralization process is instituted in bone matrix subsequent to bone matrix formation and by a quite separate mechanism. During this process the mineral ions mainly replace the matrix water, volume for volume, as they concentrate in the organic matrix focally and form hydroxyapatite crystals. These ions must diffuse into the matrix during the mineralization process and not significantly before.

This organic matrix mass produced by the osteoblasts has a high water content. The extracellular, extravascular fluid is conceived to form a water bridge from the vascular space right into this newly formed bone matrix. Thus, rapid diffusion of ions, such as those of calcium, is possible prior to matrix mineralization and during matrix mineralization. This diffusion slows as full mineralization is approached.

NICHOLS: I would like to start a discussion on this point. Your theory would seem correct, except that it must be remembered that the matrix is laid down by cells in a fluid atmosphere which contains ions; suddenly, something happens so that these ions aggregate into crystals. It seems to me that at this time one of several things may have happened. It could be that it starts very full of water, as your studies show, and something pumps out the water, leaving the silt to accumulate to the point where the mineral precipitates. A second possibility is that something is happening to that matrix which is modifying it *in situ,* or maturing it. There might be a crosslinking or some extracellular enzymatic processes going on (refs. 31 and 32). The third possibility is that the cells located at the site "get the word" and start pumping ions into the area, instead of their pumping water out of the area. It seems to me that one should believe in one of these three possibilities.

BÉLANGER: There may be a possibility also that a local change in the pH would be attributable not to the collagen but to the rest of the organic matrix, particularly the mucopolysaccharides. This is known

by the histochemical approach. The mucopolysaccharides that are close to the surface are mostly of the acidic variety, while those more centrally located seem to respond better to methods such as the periodic acid-Schiff, which indicates a neutral variety.

NICHOLS: Could you define what you mean by central and peripheral?

BÉLANGER: Yes. What is away from the osteoblast I call more central, and what is closer to the cell I call peripheral. In this bone, which Dr. McLean calls "prebone," are found a number of these acidic-staining mucopolysaccharides. This is like the situation in young cartilage; calcification cannot occur in this area because of the environment and the low pH. But as one moves away from this area and the cells become older, the tissue can make no more of them. When the cell is older and more mature, it acquires equipment to make other types of mucopolysaccharides; consequently, I think Dr. Robinson is right to think that collagen is far from being the only factor involved in the secretion of the cells away from the surface.

NICHOLS: When you say "more acidic," do you mean there is more bound sulfate at the cell surface?

BÉLANGER: This is one type. This is one way of defining these typical mucopolysaccharides. Another way of looking at them is that they would have other acidic groups apart from sulfates.

NICHOLS: Then you have to explain why the mucopolysaccharides become neutral.

BÉLANGER: I cannot explain that. I do not say that they do become neutral. But if this environment is constantly changing, it is changing under the influence of the cells which are located in one part or another of the bone. The cells at the surface possibly can make more mucopolysaccharides with sulfate than the cells which are deeper inside; the osteocytes which, because they are more mature, can now make more of another type which is more neutral, and will change from one area of bone to another.

NICHOLS: I think Dr. Howell has some data which bear on this subject.

HOWELL: Dr. Nichols was referring to our pH measurements on an essentially extracellular fluid phase sampled from normal and rachitic rats *in vivo* by a micropuncture technique to be described later. The fluid samples, about 20 mμl, have been shown to originate from the hypertrophic cell cartilage; that is, predominantly, we believe, from or close to histologic sites of calcification. With precautions to prevent loss of CO_2 the pH is measured directly with microelectrodes on such fluid sampled before and after various measures to promote calcification. Prior to healing, pH recordings in the fluid have been 7.45 to 7.55; during healing these readings increased to 7.50 to 7.58, the same as for fluid aspirated from normal cartilage plates

or from perichondrial "lymph" on rachitic cartilage plates. We think that our model of endochondral calcification may have an important bearing on mineral transfers in bone matrix.

HOLTZER: Is not this point leading up to something; namely, that the cell knows what is top and bottom and what is right and left? The polarization of the osteocytes is exceedingly relevant. It is clear that the cell does not want to make collagen in the area facing the crystallization zone. In short, the whole system is spatially oriented. The cell is commanding and determining where and when things are to occur both inside and, probably indirectly, outside the cell. To visualize this as simple precipitation in an ionic cloud would be denied by that very photograph (fig. 14), although later you might find that the cell decided that what is left field will not be right field. In short, when the cell is completely surrounded by matrix, something must happen to that cell and it says, "Now instead of sending collagen off to the right I am going to send it, or its precursor, off to the left."

NICHOLS: I think your point is very well taken, but there is another factor that I would like Dr. Bélanger to comment upon. I also think that there is a change in pH, and I should have included it. I wonder what role the glycolytic metabolism of these cells may play in this whole process. One can explain the failure of mineral precipitation of nucleation by simply invoking a lower pH around the cells without involving sulfation at all. All that is needed is a cell that happens to excrete a lot of organic acid, which we now know these cells do.

ROBINSON: I would like to complete the rest of the picture of this matrix mass surface and point out the roughness factor of the periphery of the matrix mass available to extracellular fluid on the walls of lacunae and canaliculi.

Figure 15 shows an osteocyte in a lacuna in a quite completely mineralized mass of bone matrix. This osteocyte is not very close to a periosteal, endosteal, or vascular canal surface; it is quite deeply buried. Osteocytes in such a position show atrophy of their cytoplasmic organelles; e.g., endoplasmic reticulum membrane system and mitochondria. The cells occupy less of the lacunar space. The matrix bordering the lacuna is apt to be more completely mineralized, although along one edge of this lacuna some unmineralized collagen fibrils are present. The extracellular fluid space between the cell and the bone matrix wall of the lacuna is greater than that about a newly buried surface osteocyte. (See fig. 12.) Note that at the electron-microscope level of tissue organization, the crystals produce a fairly rough and irregular surface along the periphery of the lacuna.

The protoplasmic-cell extensions can be seen proceeding out from the cell body toward the bone matrix, and one of these in figure 15 can be observed particularly well. The protoplasmic extensions do not

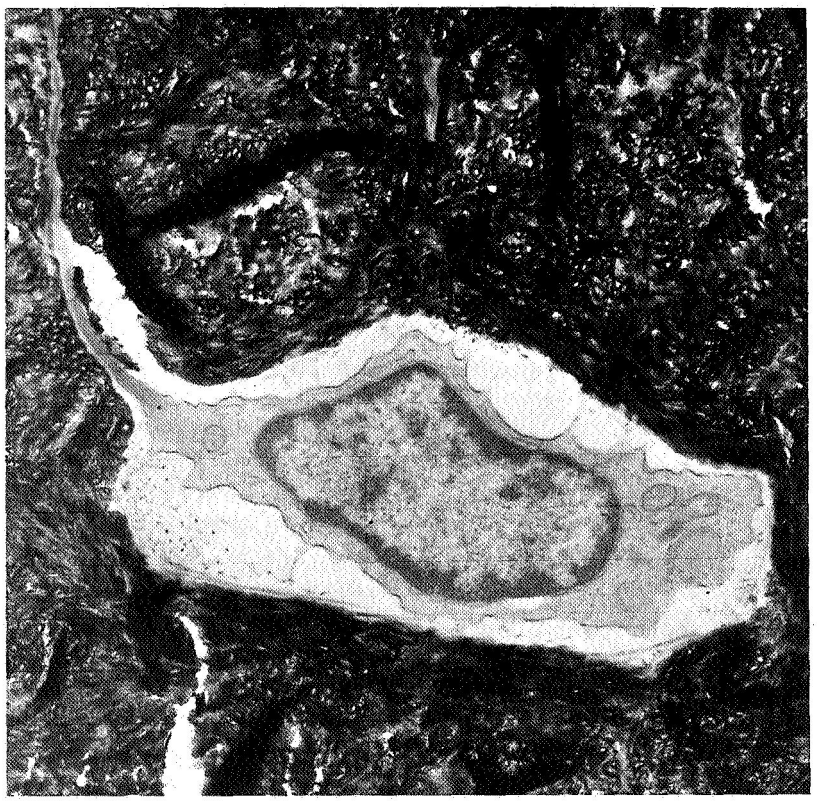

FIGURE 15. Electron micrograph of a deeply buried osteocyte in the periphery of a well-mineralized osteon in the midcortex of the diaphysis of the femur of an adult dog. These osteocytes, unlike the surface osteocytes illustrated in figure 12, show atrophy of the rough endoplasmic reticular membrane system and a marked reduction in number of mitochondria. Unmineralized collagenous fibrils are seen along the periphery of this lacuna, although unmineralized matrix is near minimum in this particular situation. The cell extension can be seen passing up to the top of the figure on the left. Approximately 6780 ×.

completely fill the canals in which they lie; there is always a small extracellular space between the plasma membrane that surrounds each one and the mineralized matrix wall of the canal. Baud (ref. 33) also found this space between mineralized matrix and the tube of cell membrane in canaliculi.

CURREY: This is not a fixation artifact? Can you be sure of that?

ROBINSON: I do not think this is an artifact. Lipp (ref. 34) in extensive light-microscope histochemical studies, demonstrated a sleeve of material in this region of canaliculi between cell membrane and bone matrix which had a high mucopolysaccharide content.

Figure 16 is a cross-sectional view of several canaliculi in quite fully mineralized bone matrix. It is interesting that one often sees two protoplasmic extensions in one canal in canine bone. We have searched for the junctions between the protoplasmic processes of osteocytes, and osteocytes and osteoblasts in canaliculi, and we have observed several of these (ref. 25). We have seen no specialized areas, such as desmosomes, on abutting plasma membranes of the two bone cells, but we have seen such specialized regions of cell membrane at the contact areas of endothelial cells. (See fig. 128.)

FIGURE 16. Fully mineralized bone matrix from the cortex of dog femoral diaphysis. The canaliculi are obvious. Many contain two cell extensions. The extracellular fluid space about these extensions is the clear white space between them and the black mineralized matrix. The collagen periodicity is emphasized in this very thin section by tiny bone crystals in the fibrils. Fascicules of larger and more compactly arranged crystals can also be seen paralleling the fiber axis; they are thought to be between the collagen fibrils. Approximately 12 000 ×.

Note the roughness of the surface of the canalicular wall produced by the little crystals in the periphery of the mineralized bone matrix. On the other hand, one should note that the mineralization of the bone matrix extends right up to the edge of the bone matrix on these canal walls, and unmineralized collagen fibrils are not observed along the walls of these canals in normal dog bone. However, along the walls of haversian canals and lacunae some unmineralized bone matrix is usually seen.

In contrast to this region of quite full mineralization of bone matrix in the middle of the cortex of a dog femoral diaphysis, I should like to return to Dr. Cameron's electron micrographs (figs. 11 and 12), where one can observe newly formed bone matrix very well. They happen to be from the surface of a metaphyseal trabecula of a growing rat, but they are good examples of partly mineralized bone matrix. In the areas of very incomplete bone matrix mineralization between foci of mineralization, many of the crystal surfaces are available. However, when mineralization is quite complete, we believe that the surfaces are no longer available because they have been shut off from rapid diffusion of calcium ions by, for example, the very fact that the water bridges necessary for the transit of these ions have been obliterated. However, in these incompletely mineralized areas, the water bridge is still open between vessel and crystal surface.

It is in these regions that tetracycline molecules and calcium ions are known to be deposited. We feel that the mechanism is quite simple; namely, that as long as a water bridge extends to the crystal surface, then the ion or molecule that has some chemotactic attraction for the crystal surface can reach the crystal surface until mineralization approaches completion. In other words, even just the surface of the mineralized matrix is a hotspot, but it is only a hotspot in a shallow sense. When very incomplete mineralization extends deeply enough through a bone matrix region, then you have water bridges in depth to all of the crystal surfaces, and you have what is known as a hotspot in autoradiographs as viewed in the light microscope.

HOLTZER: In figure 12, must you not postulate that there is actually an inhibitor here? I am amazed to see how close the crystalline area approximates the cell membrane of the cell top. There is a collagen layer, presumably, on the cell bottom.

ROBINSON: This cell used to be an osteoblast, and it is being hemmed in. This cell is creating a new matrix in this region.

HOLTZER: Yes. There must be a zone that actually cannot calcify.

URIST: What is the composition of the osteoid seam?

ROBINSON: It is collagen, water, and mucopolysaccharides.

URIST: Is this area filled with crystalline calcium phosphate? Is

it possible that this space could have been filled with noncrystalline calcium phosphate?

ROBINSON: I would like to postpone an answer to that; however, I will tell you what I feel.

We held up one paper for publication for a long time to test this point. After injecting calcium tracer into the heart of a rat and sacrificing the rat, we took slices of the tibia. We sacrificed at two time intervals, one at 2 minutes and another at 2 hours. The tracer calcium, we would think if it were going to such a pool of ions, would be quite readily soluble in the fixatives or in a fluid which is used in a dehydration bath.

URIST: Was the tissue fixed in osmic acid?

ROBINSON: No; in glutaraldehyde.

URIST: What was the pH of the solution?

ROBINSON: We never use a fixative with a pH below 7.2 whether we use glutaraldehyde or osmic acid. Both are buffered. Incidentally, if phosphate buffers are used some very peculiar pictures are obtained, so you must still be careful not to use phosphate buffers.

URIST: What kind of peculiar pictures?

ROBINSON: We start to see a great deal of mineralization in this area of unmineralized collagen.

URIST: The question is, Is there a physiologic osteoid? Does this physiologic osteoid represent collagenous matrix that contained noncrystalline calcium phosphate? The significant statement that you have made about the pH of your fixative would suggest that if such a thing does exist it would be soluble at pH 7.2. Are there calcium phosphates that are soluble at pH 7.2?

Electron micrographs do not answer Dr. Bauer's question about whether mineral *in vivo* is or is not crystalline. I think you have shown, and I agree, that bone mineral is crystalline, but I wonder if a noncrystalline material might occupy the osteoid seam. In addition to such factors as pH of the solution, is it necessary to take into consideration such factors as leaching of loosely bound deposits of mineral?

ROBINSON: If this substance exists in any concentration, one would expect that it would show up as greater density in this area. Therefore, we did this experiment that I just described. It is true that when you take sections of the tibia at 2 minutes, collect all the fixative dehydration baths, at the end of that dehydration take the remaining sample and demineralize it, put the solutions onto planchets, and count the total radioactivity of the solution, you find that in the 2-minute specimen you lose about 16 percent of the total radioactive calcium. At 2 hours, it is less than 1 percent.

In view of this experiment—and admitting that this is only one

experiment—I do not feel that the preparation method washes out a significant amount of calcium from that space.

URIST: Have you done an experiment in which you have measured the calcium content of the fixative?

ROBINSON: Yes; and also measured that of the total specimen.

URIST: So that you would account for all the calcium that was there, and you would account for the calcium that could have come off in the fixative; and thereby, you feel that you have excluded the possibility of noncrystalline calcium phosphate in the tissues?

ROBINSON: No. I do not feel that we are losing any significant quantity of calcium, because at 2 minutes after injection into the bloodstream there is still a good deal of the calcium in the extracellular fluid and in the vascular and marrow spaces; this calcium has not yet reached a crystal nucleation locus or the surface of a crystal in the bone. The reason we performed this experiment was that we had noted that as early as 2 minutes after the injection of the isotope, it was largely associated on autoradiographs with the crystalline material that we see on the periphery of the mineralized bone matrix masses, and not in that area between the cells and the crystals. I cannot say that we do not have a load of calcium, perhaps, attached to a mucopolysaccharide in this area between the bone cell and the bone crystals in the mineralized bone matrix; but I think this experiment counteracts to some extent the arguments that we are washing out a large quantity of calcium and that we are not getting a good representation of what really occurs in combined autoradiographs and electron micrographs in regions of mineralizing bone matrix.

URIST: I do not think we are inquiring about a large amount of calcium phosphate. I am inquiring about whether there is a critical transitional phase that is leached out of the tissue.

COPP: I think that you are both right. There could be calcium and phosphate in the space between the cell and the crystals which could be washed out, but we do not know how much.

ROBINSON: Obviously, calcium ions have to pass through the space from the vessel to crystal nucleation sites in bone matrix, but we doubt that calcium is concentrated in large amounts in the bone cells or throughout the unmineralized bone matrix prior to its demand at crystal nucleation sites in specific regions of the mineralizing bone matrix.

PRITCHARD: Some years ago Horning (personal communication), using microincineration, found a surprising amount of calcium in osteoid. I remember thinking there must have been a mistake; but it was true.

COPP: Obviously this is a transitional stage.

URIST: This question is a very important part of the subject of

calcium homeostasis. A recent paper by Kashiwa (ref. 35) purports to show that with the dye, glyoxal bis(2-hydroxyanil), known as GBHA, it is possible to demonstrate calcium in noncrystalline form in the proteinaceous material in the cytoplasm of osteoblasts and osteocytes. If this is true, it means a whole new area containing a small, but critical, fraction of the total body calcium is present in bone cells. The amount present in osteoid seams is not revealed because the stain does not fix calcium phosphate complexes or apatite.

Dr. H. K. Kashiwa was invited to the Bone Research Laboratory at UCLA to give instructions on his method, and we learned that he was using high concentrations of GBHA to stain whole mounts of the bone of the rat calvaria. I made thin sections of undecalcified bone and preparations of tendon with and without tendon-calcium ion complexes; I stained them by Kashiwa's technique, except with only one-tenth of the concentration that he employed, and obtained the photomicrographs shown in figures 17(*a*) and (*b*), and 18(*a*) and (*b*).

The dark-red staining, insoluble calcium–GBHA complex could be observed in the cytoplasm of osteoblasts and osteocytes; the osteoid seams and the calcified intercellular matrix were unstained (fig. 17).

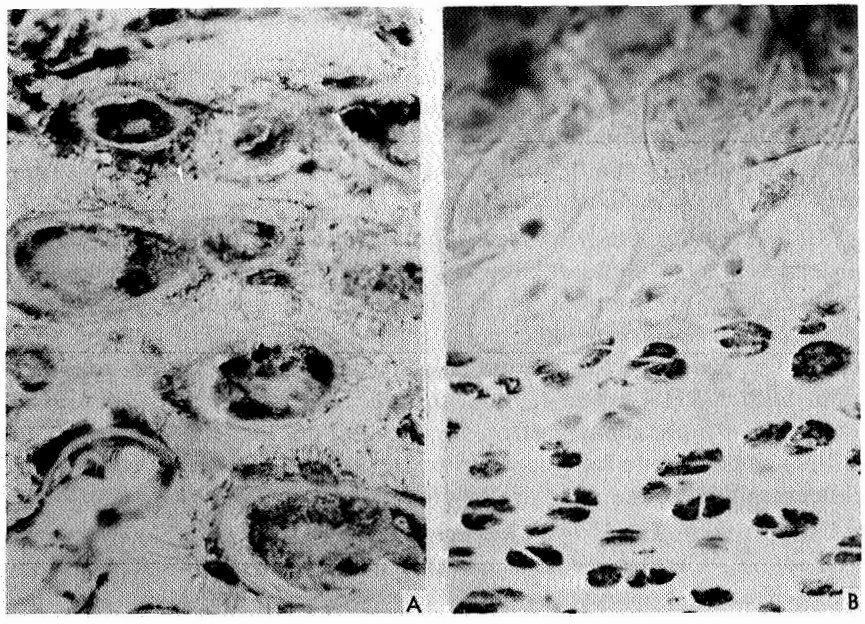

FIGURE 17. Photomicrographs of undecalcified sections of (*a*) tibial shaft cortex and (*b*) epiphyseal cartilage from a newborn rabbit. The sections were stained with a dilute solution of GBHA.

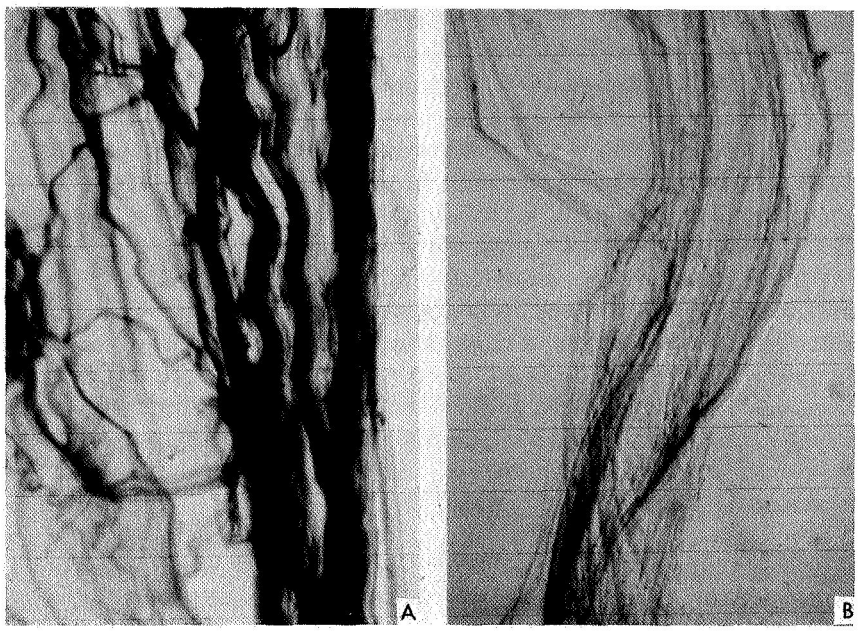

FIGURE 18. Photomicrographs of rat-tail tendon treated with (*a*) $CaCl_2$ and (*b*) $CaCl_2$ and phosphate buffer. The sections were then stained with GBHA.

The GBHA also stained the cytoplasm of chondrocytes (fig. 17(*b*)). The chondrocytes may be secreting a fibrous protein–calcium complex similar to that shown in figure 18(*a*). The calcium in uncalcified cartilage matrix or apatite in cartilage matrix is not available for reaction with GBHA under these conditions.

Rat tail tendon, treated with 5 millimoles per liter of $CaCl_2$, is stained a deep red by a dilute solution of GBHA (fig. 18(*a*)). The tendon-collagen-calcium complex binds GBHA in an insoluble precipitate. Tendon untreated with calcium is unstained or weakly stained with GBHA. The tripartite tendon-calcium-phosphate complex does not stain with GBHA when tendon collagen is treated first with $CaCl_2$ and then with phosphate buffer, 5 millimoles per liter (fig. 18(*b*)). The affinity of phosphate for calcium is greater than that of GBHA when low concentrations of the stain are employed.

COPP: I think Dr. Howell has some data on this point.

HOWELL: Cartilage data show this increase in calcification up to the ossification process.

COPP: Dr. Robinson's magnificent electron micrographs of bone have given us some idea of the problems and interactions between bone mineral and extracellular fluid. We have found, for example,

that the plasma calcium dropped 30 percent after thyroparathyroidectomy in the rat, and there was a corresponding drop in exchangeable bone calcium (ref. 36).

BAUER: That is your computation from the accretion rate?

COPP: The accretion rate also changed.

BAUER: I wonder if there is any experimental evidence that alterations in exchangeable bone calcium do occur. You say that the accretion-resorption rate did not change. However, I think it is right to say that no one has seen a change in the exchangeable amounts without some change in the accretion-resorption, so it is more of a concept than actually based on evidence.

COPP: I do not agree. When calcium is injected into a thyroparathyroidectomized dog, as in figure 19, a new equilibrium is reached at a higher plasma calcium level. There is a corresponding increase in the labile bone calcium pool, which we estimate to be 50 to 80 mg/kg.

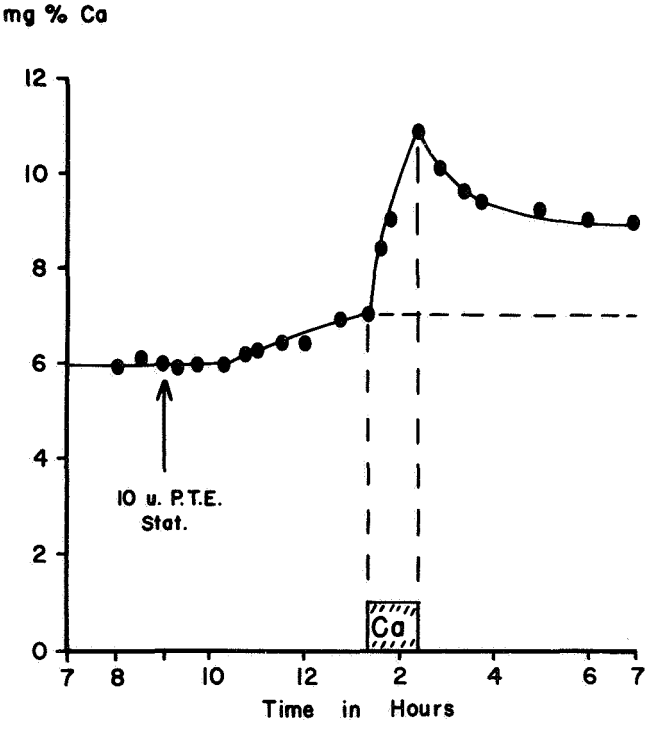

FIGURE 19. Increase in the equilibrium level for plasma calcium after intravenous infusion of calcium (10 mg/kg) into a dog thyroparathyroidectomized 1 week earlier. The dog also received a dose of parathyroid extract sufficient to raise the plasma level 1 mg/100 ml, as indicated by the broken line.

BAUER: I had estimated it to be 5 grams per 100 units.

COPP: No. We estimate the pool on the basis of body weight, and it is interesting to note that our estimate agrees quite well with your estimates of exchangeable calcium as measured with radioactive isotopes.

CURREY: I would like to ask a question about this labile pool which I do not quite understand. It seems to me that the labile pool is a surface phenomenon; therefore, why do you say that the amount in the labile pool is a measure of change?

COPP: Your point is well taken. The pool really represents a volume capacity; assuming arbitrarily that the concentration of calcium in this labile bone storage pool is the same as in plasma, we would estimate a pool size of 500 to 800 mg/kg body weight. At a normal plasma calcium level of 10 mg/100 ml, the amount of calcium in this pool would be 50 to 80 mg/kg. Assuming the capacity of this pool is unchanged, an increase of 2 mg/100 ml in plasma calcium would correspond to an increase to 16 mg/kg in the calcium in this pool.

There are two distinct and different points of view with respect to the exchange between the calcium in bone and the calcium in extracellular fluid. We may not be able to resolve these differences in the time available, but they should be clearly stated.

Our point of view is that those crystals of bone salt which are accessible to the circulation act like an ion exchange column, with calcium on the surface of the crystals exchanging with the calcium in the surrounding fluid by a purely physicochemical process. This would correspond with the almost universal uptake of ^{45}Ca around all the vascular channels of bone observed by Dr. Rowland a few minutes after injection of the isotope. As with any ion-exchange column, if the ionic concentration of calcium in the fluid increases, the concentration of calcium on the crystal surface will increase. This, we feel, corresponds to our labile bone storage pool for calcium. The other view holds that there is a regulating biologic membrane separating all bone mineral from the surrounding fluid; the level of calcium in the fluid depends on this membrane and the effect that the calcium active hormones have on it. In both cases, it is agreed that there are biologically active and hormone modified processes of accretion and resorption which determine the ultimate calcium balance between bone and body fluids.

ARNAUD: I do not think that we have enough information or the techniques to distinguish adequately between these processes.

COPP: Dr. Talmage would like me to mention the parathyroids, which he thinks are related to calcium homeostasis. Their importance was indicated by the classic work of Hastings and Huggins (ref. 37). The brilliant hypothesis of McLean (ref. 38), and the experiments

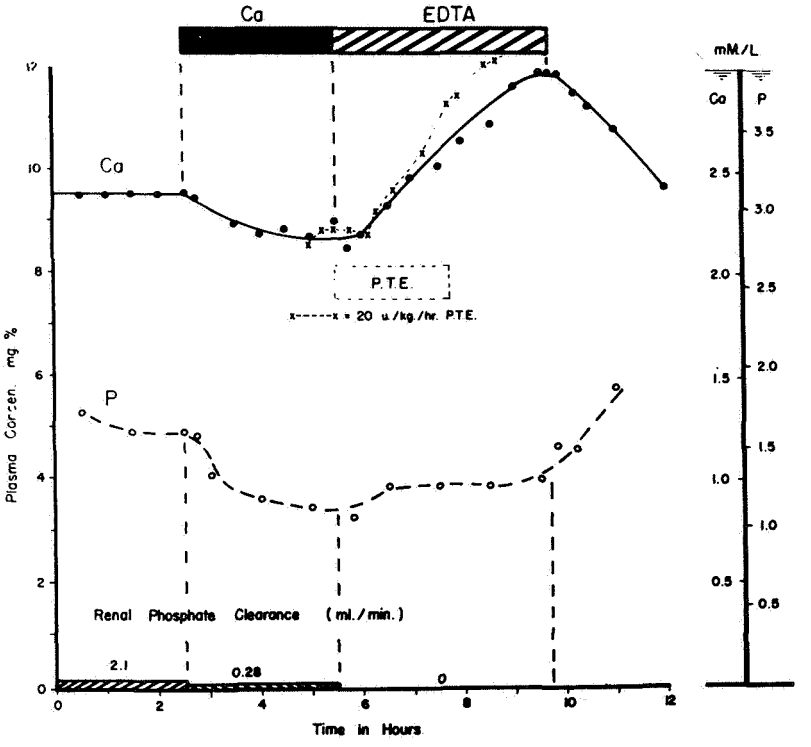

FIGURE 20. Typical response of systemic plasma calcium and phosphate levels to perfusion of parathyroid with blood to which calcium or EDTA has been added. Dotted line shows changes in the same dog when parathyroid extract (PTE) (20 units/kg/hr) was injected for a 3-hour period 6 hours after thyroparathyroidectomy. [From ref. 39; reprinted by permission of the publisher.]

of Copp and Davidson (ref. 39), clearly showed that this was accomplished by negative feedback control. When the thyroid and parathyroid glands of a dog (fig. 20) were perfused with blood low in calcium, there was a rise in systemic plasma calcium which closely resembled the response obtained in the same animal with continuous intravenous infusion of 20 units/kg/hour of parathyroid extract. This is approximately 200 times the maintenance output of the parathyroid hormone as determined by us in previous experiments (ref. 13). These values are generally in line with the observations of Sherwood et al. (ref. 40) and Care et al. (ref. 41) on parathyroid hormone production in cows, sheep, and goats in which the glands were stimulated or inhibited by injection of EDTA or calcium. The hormone in plasma was determined by radioimmunoassay.

In our studies on perfusion of the isolated glands and in studies

on increased mobilization of calcium during calcium infusion, as shown in figure 21 (ref. 42), it is apparent that a fall of as little as 10 to 15 percent in the level of calcium in the blood passing through the gland is sufficient to stimulate hormone production.

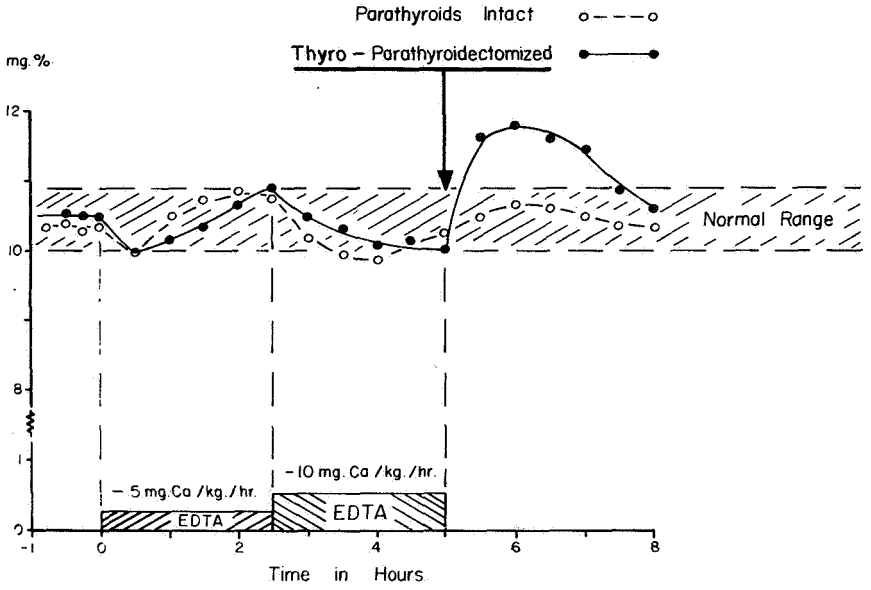

FIGURE 21. Effect of EDTA infusion on plasma calcium level in the same animal with intact glands (broken line) and after removal of the thyroid and parathyroids immediately after EDTA infusion as indicated by the arrow. [Adapted from ref. 42; reprinted by permission of the publisher.]

Dr. Raisz has done some beautiful studies on the effect of the calcium concentration on parathyroid cells in organ cultures (ref. 43). Dr. Raisz, would you comment on this work?

RAISZ: I think we are now at the point where an elegant servo-analysis of calcium homeostasis could be achieved if we could get precise-enough data to satisfy mathematicians. One of the difficulties in a biologist's work is that he can never satisfy the mathematical demands for large numbers of points, but he can make an attempt. I think a model, on the basis of which we can make a specific analysis, is available. I would like to make the simplifying assumption—which we can debate—that the controlling system in calcium regulation is bone resorption and not bone formation. Bone formation is likely to be controlled by matrix synthesis, by hormones which regulate bone growth, and by local factors which determine the structures of bone; one might call these the orthopedic functions of bone.

NICHOLS: When you speak of "bone resorption," do you mean both mineral and matrix or just mineral?

RAISZ: I will talk about mineral, but it is obvious that one cannot remove adequate amounts of mineral from bone without getting rid of some of the matrix.

URIST: Are you referring to second-to-second control of calcium homeostasis?

RAISZ: No.

URIST: Is matrix formation involved in second-to-second control?

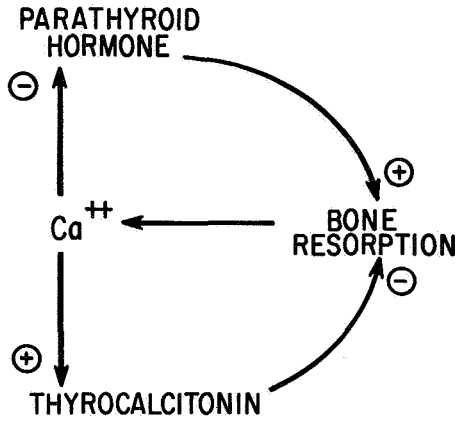

FIGURE 22. Illustration of the simplest model for feedback regulation of blood calcium ion concentration in which bone resorption is the major controlling system. In addition to the feedback control of parathyroid hormone, the possible role of thyrocalcitonin is indicated.

RAISZ: No; it is not.

URIST: I would think certainly hour-to-hour control required matrix formation, and that you are correct in assuming that second-to-second control is a function of the mineralized surfaces.

RAISZ: Given bone resorption as a controlling system and calcium ion as the controlled system, we have, in the McLean hypothesis, negative feedback control through parathyroid hormone which is constantly secreted and stimulates bone resorption. These are all things we have known since the 1920's. (See fig. 22.) Now we can ask what kind of control this is. We have only fragmentary evidence at present, but as investigators get better at studying the way in which the parathyroid gland responds to calcium, they can ask questions of the

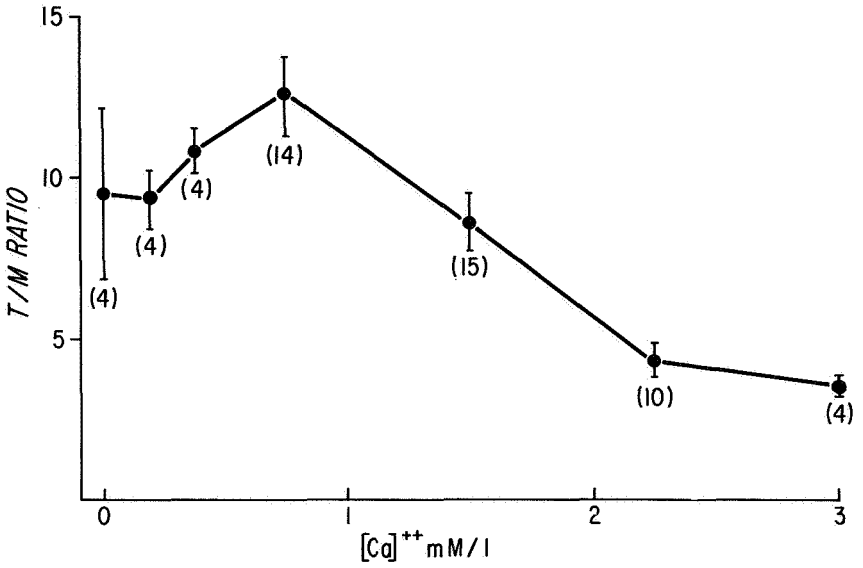

FIGURE 23. Effect of calcium concentration on tissue/medium concentration ratio of ^{14}C-alpha-aminoisobutyric acid after 2-hour incubation. Points are mean values with SE. Number of observations is given in parentheses. [From ref. 44; reprinted by permission of the publisher.]

kind which have been asked about such feedback mechanisms as the carotid sinus control of blood pressure. For example, is there an amplified proportional control whereby small changes in calcium level cause proportionately large changes in parathyroid hormone output? I think we can assume that parathyroid hormone has two ways of functioning. One is an amplified proportional feedback which responds to minute-to-minute changes in serum calcium concentration, both by changes in secretion and changes in hormone synthesis. The second is an integral response which requires growth of new cells or possibly involves activation of cells which have been in a quiescent condition. These are probably general phenomena of endocrinology. This is illustrated by the feedback control of the uptake of alpha-aminoisobutyric acid (AIB), a nonmetabolized amino acid analog which is actively transported into the cells by the system responsible for the uptake of the natural amino acid, glycine (ref. 44) (fig. 23). These data were obtained from isolated rat parathyroid glands incubated for 2 hours at varying calcium concentrations. Normal calcium concentration would be about 1.5 mM. Between ½ and 1½ times this concentration, there is a steep curve relating AIB uptake inversely to calcium concentration. With lower or higher concentrations beyond this range,

there is little further change in AIB uptake. These data, then, follow an S-shaped curve which (while not as clear cut as the data for the carotid sinus regulation of blood pressure) does indicate a proportional response of the parathyroid to calcium concentration which is amplified around the normal calcium concentration of blood. To prove this, extensive data on the effect of calcium on the rate of secretion *in vivo* will be required. Assuming that such a response exists, maximal stimulation occurs at half normal calcium concentrations; the only way in which the parathyroid can respond further to prolonged stimulation would be to increase its functional tissue mass. Figures 24 to 26 illustrate such a response. The model is the vitamin-D-deficient rat in which, because of end organ failure, i.e., the inability of bone tissue to respond to parathyroid hormone, one can see prolonged hypocalcemia over many days (ref. 45). With hypocalcemia there is an

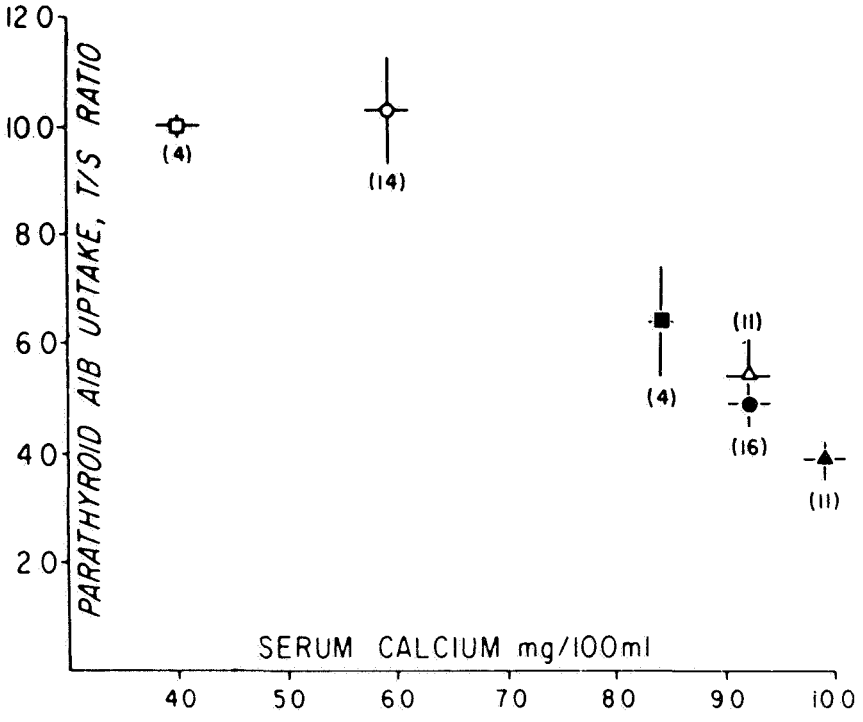

FIGURE 24. Relationship between serum calcium concentration and parathyroid uptake of AIB expressed as the ratio of AIB concentration in parathyroid tissue water to that in serum (T/S ratio) at equilibrium 24 hours after AIB injection. Each symbol represents mean and SE for both serum calcium concentration and parathyroid AIB uptake. Figures in parentheses represent number of animals in each group. Symbols for different diets are indicated in figure 25. [From ref. 45; reprinted by permission of the publisher.]

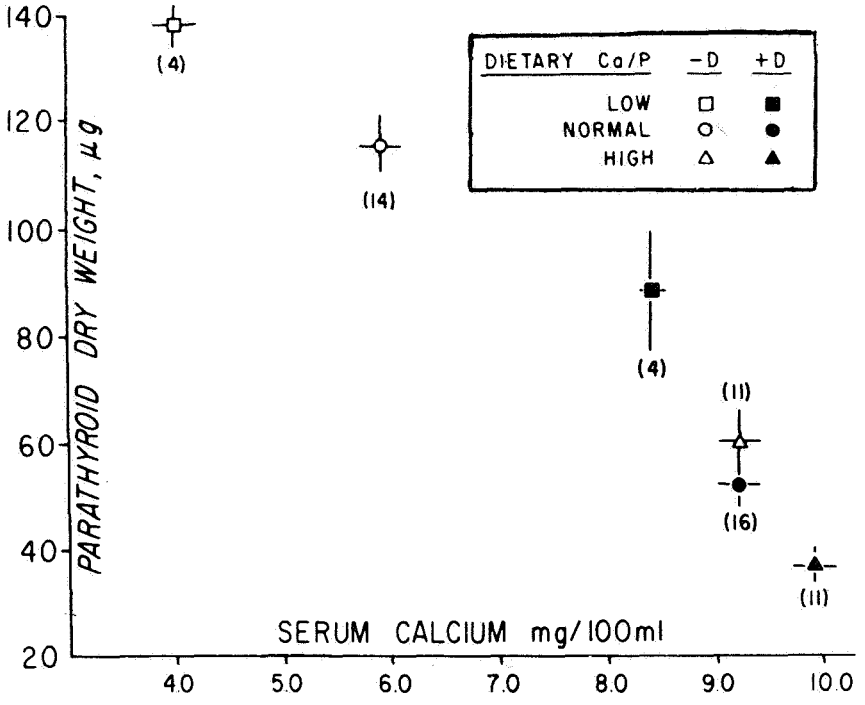

FIGURE 25. Relationship between serum calcium concentration and parathyroid dry weight. Legend indicates whether animals were on vitamin D-deficient (− D) diets or on the same diet with vitamin D supplement (+ D), 80 I.U. every 3 days. The diets were as follows: low Ca/P = 0.03% Ca and 0.1% P; normal Ca/P = 0.4% Ca and 0.3% P; and high Ca/P = 0.8% Ca and 0.1% P. [From ref. 45; reprinted by permission of the publisher.]

increase in parathyroid AIB uptake as the serum calcium falls to 6 mg/100 ml (fig. 24). With further lowering of serum calcium, achieved by combining vitamin D deficiency with a low calcium intake, the serum calcium falls still further, but there is no further increase in the function of the tissue as indicated by the AIB uptake. However, the gland can respond by getting bigger. Figure 25 illustrates the inverse relationship between parathyroid size and serum calcium for both dietary changes in calcium and for the effects of vitamin D deficiency. At the risk of overanalyzing our data, we have taken the activity of the gland, as measured by amino acid uptake, and the size of the gland, multiplied them together, and divided by body weight to obtain a parathyroid activity index. Figure 26 illustrates the remarkably good inverse linear correlation between calcium concentration and the parathyroid activity index. This linear response is a reflection of integral control. That is to say, not only is the change in serum cal-

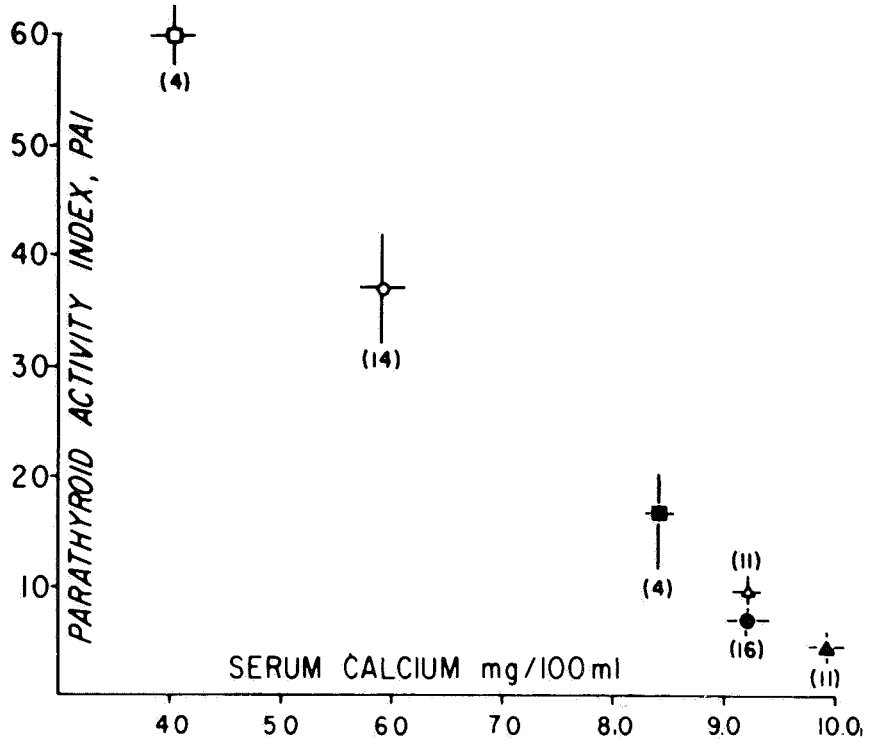

FIGURE 26. The relationship between serum calcium concentration and parathyroid activity index (PAI). Symbols are defined in figure 25. PAI is calculated as the T/S ratio times parathyroid size divided by body weight. This index combines the effect, by any given experimental procedure, on size and activity of the parathyroid and corrects for any difference in body size. [From ref. 45; reprinted by permission of the publisher.]

cium responded to proportionally but the prolonged duration of that change is able to alter the amount of functioning parathyroid cell mass. Thus, by increasing both activity and amount of tissue, we can greatly increase the total parathyroid response. I suppose there must be a limit, perhaps when the parathyroids become larger than the rat, but there appears to be a great capacity for an integral control in the parathyroids.

FREMONT-SMITH: What is your AIB?

RAISZ: Very good. Alpha-aminoisobutyric acid, which is non-metabolized. That is the amino acid transport system largely for glycine. The shocking level is up to about half normal. Normal would be about 1.5 millimoles per liter. Shocking level up to half normal has no consistent steps. Between ½ normal and 1½ times

normal shocking level, one has a deep curve relating inversely to shocking concentration.

We still need to obtain data on AIB uptake *in vivo.* I would like to see data on normal secretion *in vivo* which would show, I believe, that the greater secretion changes rapidly; that is, the proportional response to the parathyroid is amplified around the normal calcium level, just as the response of the carotid sinus is amplified around normal blood pressure, but proceeding with greater regulation toward normal.

Assuming for the moment that this is likely to be true about the parathyroid, that will answer the problem of prolonged stimulation.

HOLTZER: As you say, that is really tremendous. Have you checked that out as to the number of cells on the surfaces, for example.

RAISZ: We have shown that low calcium increases RNA synthesis in tissue culture.

HOLTZER: This ought to be DNA; that is the whole point.

RAISZ: We have done no direct DNA studies. With embryonic tissue one gets an increase in mitoses and in the number of cells. We have not been able to get many mitoses in adult rat parathyroids in tissue culture, but we know that this can happen *in vivo* from the studies of Talmage and Toft (ref. 46).

NICHOLS: I think that there is reasonable, although less direct, confirmation of these views in human material.

First, there are people with uremia in whom there are hypertrophied parathyroids which are very large indeed and, by inference, there are more cells present. Evidence is also available from such patients, which suggests that these glands revert toward normal. If a successful kidney transplant, which functions for several months, is achieved, the serum calcium of these patients returns to normal. Indeed, if they have metastatic calcification, this disappears. In the one patient I know of, who has since come to post mortem, the parathyroids were normal (ref. 47).

RAISZ: I would like to point out that the other half of the regulation still requires further study; that is, the opposite effects of thyrocalcitonin. I do think that the same kind of approach can be made to studies of thyrocalcitonin regulation.

COPP: The only thing I would do would be to twist it the other way around. We hope to do the experiment you mentioned, and measure the enhanced parathyroid hormone output by immunoassay during EDTA-induced hypocalcemia.

MCLEAN: Before you go on with thyrocalcitonin, I would like to add another human model to the one Dr. Nichols has mentioned. This is a situation in which there is an adenoma of one of the parathyroids, and the other parathyroid glands are exposed to hypercalcemia over a long period of time. This is being intensively studied now and it

will add a great deal to our understanding of the control of parathyroid secretion. The other parathyroids under that situation quiet down.

FREMONT-SMITH: Atrophy——

MCLEAN: I do not think you can use the word "atrophy." They subside. The surgeon looks at them and he says, "These other glands are atrophied." They are being studied now with the electron microscope and more information is coming out about what is going on over a long period of time so that integral control may be observed.

FREMONT-SMITH: You say they quiet down. A sort of functional atrophy—an atrophy of function.

MCLEAN: They function at a lower level. I doubt that they ever cease functioning completely.

RAISZ: There are substantial data from Dr. Sanford I. Roth of Massachusetts General Hospital, not only on the functional atrophy of the human parathyroid gland but also on the effects of calcium on parathyroid morphology in tissue culture (ref. 43). Dr. Roth has inferred from electron-microscope pictures (we should remember that one can only guess at the sequence of events with such static pictures) that there are cycles of hormone synthesis and secretion in the parathyroid cell. Dr. Roth thinks these cycles are slowed down in high calcium and speeded up in a low calcium environment. This kind of effect can be seen both in tissue cultures of rat parathyroids and in human material.

COPP: Dr. Talmage, would you like to say something before we leave the parathyroids?

TALMAGE: One particular aspect of parathyroid action has bothered me. This aspect was illustrated recently by Harrison and Harrison (ref. 48) in a paper relative to vitamin D effects on calcium absorption by the gut *in vitro*. It was not their study of vitamin D that bothered me. However, in their discussion they assumed that parathyroid action on bone was extremely slow. This assumption was made as if it were a generally accepted fact. I think it is very important for us to emphasize here that parathyroid is a very rapidly acting hormone. It produces effects on bone in minutes, not in hours. We should endeavor to counteract the idea of this slow action which is so prevalent in the literature. Apparently this idea results from the fact that small calcium changes are difficult to identify due to the large pool size of extraosseous calcium.

Unfortunately, Dr. Copp's recent emphasis that thyrocalcitonin is faster acting than parathyroid hormone can add to this misconception. While he is speaking in relative terms, the description of parathyroid hormone in this matter has aided the erroneous concept prevalent in the literature. I believe this is one of the most important aspects of the hormone to be kept in mind.

URIST: Does the rate of the response depend on the age of the animal?

TALMAGE: No.

COPP: I would like to comment on this, since you brought the matter up. I believe parathyroid hormone is fast acting, even in dogs and man, but in these species the effect persists for many hours.

TALMAGE: You are talking about the half-life of the hormone after it is secreted.

COPP: I am not talking about the half-life of the hormone in circulating blood, which Sherwood et al. (ref. 40) have shown to be on the order of 20 to 30 minutes. I am talking of the duration of action, which may be for many hours.

NICHOLS: I think your point is extremely well taken. Part of the reason we think it takes so long is because we have always looked at it from the point of view of injecting parathyroid hormone into intact animals. I think Dr. Arnaud ought to speak about this in relation to his newer animal preparations. Our recent experience, using thyrocalcitonin, suggests that parathyroid hormone effects occur within an hour.

COPP: We have observed elevations in plasma calcium within 20 to 30 minutes of intravenous administration of parathyroid hormone in the dog, and there must be changes in bone which precede this elevation.

TALMAGE: Yes; but one-half hour is a relatively short time compared with 6 hours, as reported by Harrison and Harrison (ref. 48).

COPP: Well, a 6-hour delay is ridiculous.

NICHOLS: Good point. Howard Rasmussen suggested in 1961 (ref. 49) that there are effects of an injection of parathyroid hormone that last for a very long time, these being the ones on the bone mineral. Recently, we have done some experiments with thyroparathyroidectomized animals given a single injection of parathyroid hormone, looking for the effects on acid production by cells and the solubility of the mineral.* It turns out that there is a clear time sequence of these events as you would suspect; namely, that an effect on the acid production by the cells can be seen within 1 or 2 hours. This has largely faded after 12 to 18 hours; lactate production then falls below normal up to 48 hours and subsequently drifts back to normal. The mineral effect, on the other hand, comes later, lagging anywhere up to 6 to 8 hours behind the changes in acid production and this continues to increase for as long as 2 days.

RAISZ: I have contradictory data. In tissue culture one can show

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*Asher, J. D.; Nichols, G., Jr.; et al.: Unpublished observations. Some of these observations were conducted as part of a required class laboratory by members of the Harvard Medical School class of 1969.

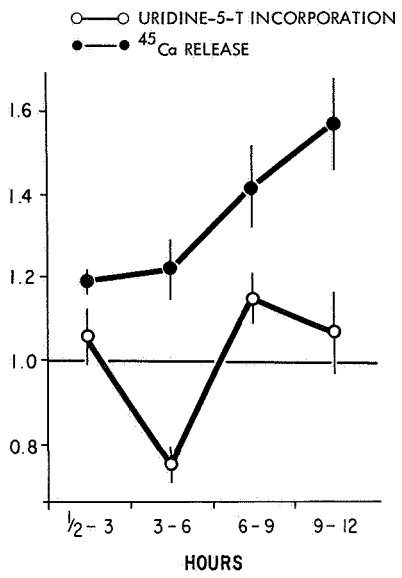

FIGURE 27. Treated versus control ratios plotted against time showing the effect of parathyroid hormone (1 μg/ml) on the release of calcium and the incorporation of uridine into RNA in paired cultures of embryonic bones. Each point represents the mean and SE of 8 to 12 pairs of cultures (6 bones per culture). Embryonic bones in which ^{45}Ca had previously been incorporated were precultured for 24 hours and then transferred to media with or without parathyroid hormone. The relative release of ^{45}Ca into the medium and uptake of uridine from the medium into the bone RNA were measured for various intervals after hormone addition. [From unpublished observations of L. G. Raisz and Ingrid Niemann.]

an apparently immediate effect on calcium release from embryonic bone. These bones are labeled with ^{45}Ca and then precultured so that exchangeable ^{45}Ca on the surface is largely removed. During the first 3 hours after addition of parathyroid hormone, there is a 20-percent increase in calcium release (fig. 27). This represents less than 1 percent of total bone calcium. This release rate continues during the first 6 hours; after 6 hours there is a sharp break in the curve and the calcium release rapidly increases for parathyroid hormone-treated bones. Hence, there is a change from an initial phase of a modest parathyroid effect on calcium release to a second phase of augmented

parathyroid effect. In these experiments we also studied the effect of parathyroid hormone on the incorporation of uridine into bone RNA. During the first 6 hours there was inhibition of uridine incorporation. This may represent the known inhibitory effect of parathyroid hormone on the osteoblasts. At about 6 hours there is an increase in the incorporation of uridine into RNA; this increase is even more marked in experiments where the tissue is pulse labeled for 1 hour (fig. 28).

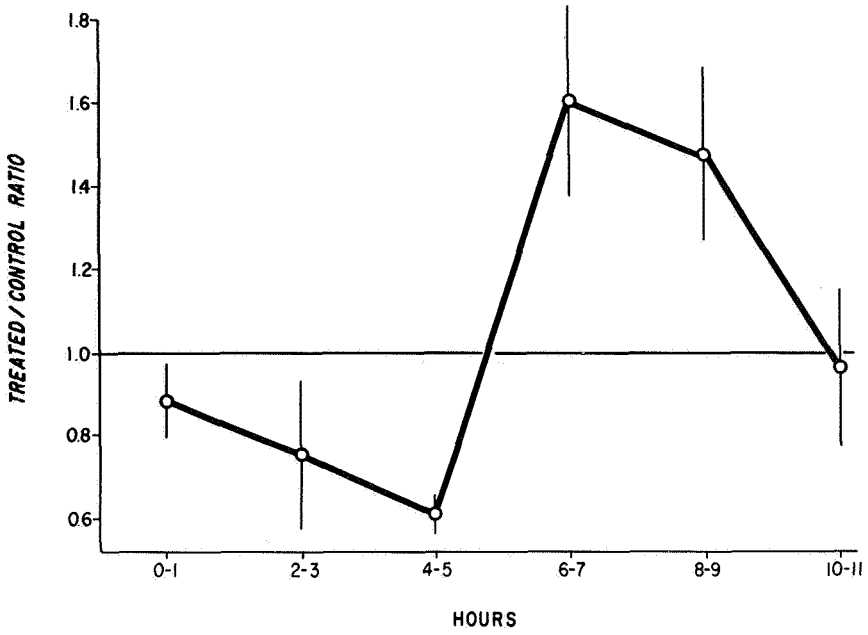

FIGURE 28. Effect of parathyroid hormone (1 μg/ml) on uridine-5-T incorporation into RNA after 1-hour pulse label. Each point represents the mean and SE of six to nine pairs of cultures (two to six bones per culture). Conditions as for figure 27. The 1-hour uridine uptake shows an abrupt increase for the 6- to 7-hour period. [From unpublished observations of L. G. Raisz and Ingrid Niemann.]

SAXÉN: Is this into the total RNA?

RAISZ: Yes. This is preliminary work; we have not been able to measure pool size or to isolate the RNA and characterize it. It does fit in with the idea that parathyroid hormone initially causes a rapid effect on calcium movement in the cells and that, subsequently, the bone responds with a change in its cell population. We can actually see this histologically after 8 to 12 hours in culture with the appearance of more osteoclasts. This then goes on to the extensive complete resorption that one can see in tissue culture.

ROBINSON: Then, is this bone messenger RNA or soluble RNA?

RAISZ: This is total RNA. We think that the 1-hour incorporation is mostly messenger RNA.

ROBINSON: Why not? Another thing is, if it affected the cell right away you would not see the effect for 4 hours, would you? You said it takes the cell that long to "tool up."

RAISZ: You see messenger RNA synthesis right away.

COPP: I would like to ask Dr. Bélanger to comment on this point, since he has some information on the cellular responses in bone to injection of parathyroid hormone. There is an immediate effect and a delayed effect which might correspond to increased osteoclast activity.

NICHOLS: Dr. Copp, Dr. Raisz said earlier that he had completely opposite data. In point of fact, they are not a bit opposite. The early mineral effects that he is seeing occur just when we see an effect on cellular acid production, both lactate and citrate. Later on, about 6 to 8 hours after injection, the passive solubility of the mineral starts to change. If I am not mistaken, this is the point where you start to get a sharp rise in calcium release. What I am saying is that it takes several hours for the surface that we were talking about to become modified by the increased cellular acid production which is induced by PTH. When you use a live tissue in your system, it is reflected by a lag in the calcium release. We get exactly the same thing when using our fragments if we keep them alive.

RAISZ: Having injected the PTE in the animal?

NICHOLS: Yes.

RAISZ: You are saying that there is a calcium effect that you could not measure, but that you could measure the effect on lactate.

NICHOLS: Looking at the steady-state distribution of calcium between medium and bone, there is an effect in the early phases around live pieces of bone. But if you look only at inactivated tissue, the effect does not appear until later (ref. 50). The important point is that the mineral, and its solubility, is modified by the change that has been going on in the metabolism of the cells.

TALMAGE: I would like to say that we have lots of RNA data not only at 1 hour but at 20 minutes, but I have an entirely different interpretation on this RNA. I do not believe this early change has anything to do with homeostasis. I would just like to say that I have these data and would like to discuss them later.

NICHOLS: Dr. Raisz says it is messenger RNA in its early phase. I do not think that is correct. I have reason to believe so, and would like to discuss the point later.

COPP: You can certainly bring up this matter later. One of the important questions is the way in which parathyroid hormone produces

its effect in normal calcium homeostasis. Dr. Bélanger has some views on this.

BÉLANGER: At this stage, I can only offer the following comments related to the present discussion.

If we look at various parts of the skeleton after endogenous stimulation of the parathyroid gland, or after parathyroid hormone, as done by Dr. Copp, we can see this. If we compare one particular region, for instance, in these two fashions, first look at it in its mineralized phase through a modified type of microradiography using alpha bombardment, we can see that the loss in density is coincidental in the various areas of bone.

On the other hand, the loss of organic material is over a wider area than the loss of mineral substance (ref. 51). These data, which we obtained in British Columbia, allowed us to think that the primary effect of parathyroid hormone was on the cells and that the immediate response of the cell was to destroy somehow or to modify the organic substance around some of these cells; the amount of destruction being far greater than the amount of loss of salt seemed to indicate that the organic effect was primary.

COPP: The osteocyte is now threatening the osteoclast.

BÉLANGER: These are two different things, two different worlds indeed.

COPP: Is there any further comment on the parathyroid?

URIST: Dr. Arnaud, your work has been mentioned several times. Do you agree with what has been said about it here?

ARNAUD: I am afraid that both the mechanism of PTH action and bone cell metabolism are exceedingly complex and the techniques available for their study *in vivo* are quite crude. The measurement of an increase in the concentration of calcium in the plasma of the PTH-treated parathyroidectomized organism, although a physiologically important effect, merely represents the end result of a series of reactions of which we know little. Recognizing this, the measurement of the time required to observe this result has been complicated by the fact that most studies have been done in animals whose thyroid glands have been left intact. Under these conditions, thyrocalcitonin secretion is not controlled and its presence might be expected to inhibit the plasma-calcium response to administered PTH. This is in fact the case. When the thyroparathyroidectomized rat is given parathyroid hormone a response can be observed as early as 30 minutes and possibly sooner. I should expect that if one could measure another specific, and more sensitive, index of parathyroid hormone action on bone, an almost instantaneous effect could be observed.

COPP: I would like to ask one question. Do you consider half an hour fast or slow?

ARNAUD: I do not think that we have the techniques to measure how rapidly parathyroid hormone acts.

COPP: It has been known for a long time that a response may occur within 20 to 30 minutes.

ARNAUD: Well, we are measuring the end result.

BAUER: Does the parathyroid hormone act in the absence of vitamin D?

ARNAUD: We have made our position rather clear about this. Our studies have shown, first, that it is extremely difficult to produce an animal which is entirely depleted of vitamin D; and second, that the osseous response, if any, of this animal to parathyroid hormone depends upon the degree of vitamin D deficiency induced (refs. 52 to 54). Harrison et al. (ref. 55) and Harrison and Harrison (ref. 56) were the first to demonstrate this dependency relationship. There has been work reported which is contradictory (refs. 57 and 58), but the major portion of it can be questioned on the basis of the adequacy of vitamin D depletion.

The important concept that has come out of our studies of vitamin D deficiency and its interrelationship with parathyroid hormone action is that parathyroid hormone appears to have its characteristic effect on the renal tubule in the vitamin-D-deficient animal, whereas it does not have its characteristic effect, or at least the degree of effect, on bone.

This is interesting in that studies done by Rasmussen et al. (ref. 59) and by Tashjian et al. (ref. 60) with inhibitors of protein synthesis seem to be analogous to the results observed in vitamin D deficiency. The influence of parathyroid hormone on the kidney of the actinomycin-D-treated and the vitamin-D-deficient rat is qualitatively the same as that of the normal animal, whereas its effect on bone is, in large part, blocked.

TALMAGE: I would like to disagree with that for the record. Actinomycin D does not specifically block parathyroid action in bone, at least relative to the control of plasma-calcium levels. It is true that this drug drops calcium levels and appears to inhibit the action of injected hormone, but this is a result of the general effect of this drug on all cells. We feel that we have demonstrated convincingly that the hormone is still functioning in animals under heavy actinomycin D treatment.

COPP: If we can continue this discussion later, I would now like to complete the endocrine contribution to calcium homeostasis.

We will now turn our attention to the thyroid, the other endocrine gland directly involved in calcium homeostasis. In our original experiments (ref. 61) in which we perfused the thyroid-parathyroid apparatus in the dog with high and low calcium blood, we observed that exposure of the glands to hypercalcemia resulted in a prompt fall in systemic plasma calcium which was much more rapid than the fall

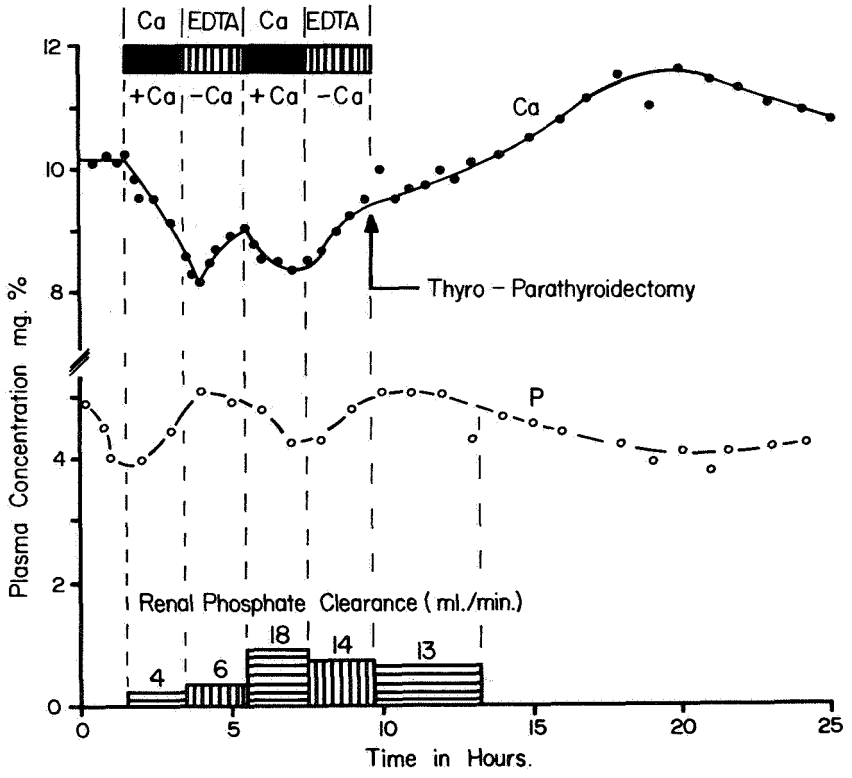

FIGURE 29. Changes in plasma calcium during successive perfusions of the thyroid-parathyroid glands in a fasting dog with blood alternately high and low in calcium. The glands were removed as indicated by the arrow at the end of the last EDTA infusion. [Adapted from ref. 42; reprinted by permission of the publisher.]

which occurred after surgical removal of the glands. In the definitive experiment illustrated in figure 29, high calcium perfusions of the glands caused a rapid fall in blood calcium, while low calcium (EDTA) perfusions caused a rise, presumably due to the release of endogenous parathyroid hormone (ref. 62). To test the current hypothesis that the effect of hypercalcemia was due to suppression of parathyroid hormone production, after establishing the responses through two cycles, we removed the thyroid and parathyroid glands. Instead of the anticipated fall, the plasma calcium rose and remained elevated for many hours. It was evident that the effect of hypercalcemia was a result of the release of a humoral agent which was responsible for the rapid fall in systemic blood calcium. Indeed, we found that the high calcium perfusates did produce hypocalcemia when injected into a second dog. These results were confirmed by Kumar et al. (ref. 63), by using ion-exchange resins to modify the calcium concentration in the perfusing

blood. We named this new hormone "calcitonin," since it appeared to be involved in regulating the level, or tone, of calcium in body fluids. While first thought to come from the parathyroids, it is now apparent that the calcitonin originally demonstrated in these experiments was of thyroid origin (ref. 64). In 1963, Hirsch et al. (ref. 65) observed that destruction of the parathyroids by hot-wire cautery caused a much greater fall in plasma calcium than that which occurred after surgical removal of the glands. They were also able to extract a potent hypocalcemic and hypophosphatemic substance from thyroid tissue, to which they gave the name "thyrocalcitonin" to indicate the gland of origin, and its possible identity with calcitonin. Care (ref. 66) has clearly demonstrated release of thyrocalcitonin by high calcium perfusion of pig thyroid, which contains no parathyroid tissue. (See fig. 30.) In collaboration with Dr. Bélanger, we have also shown that 24 hours of mild hypercalcemia (+ 10 to 15 mg/100 ml) in the sheep results in hypertrophy of thyroid cells—particularly the parafollicular light cells.

NICHOLS: May I ask if those changes were in both the parafollicular and the follicular cells?

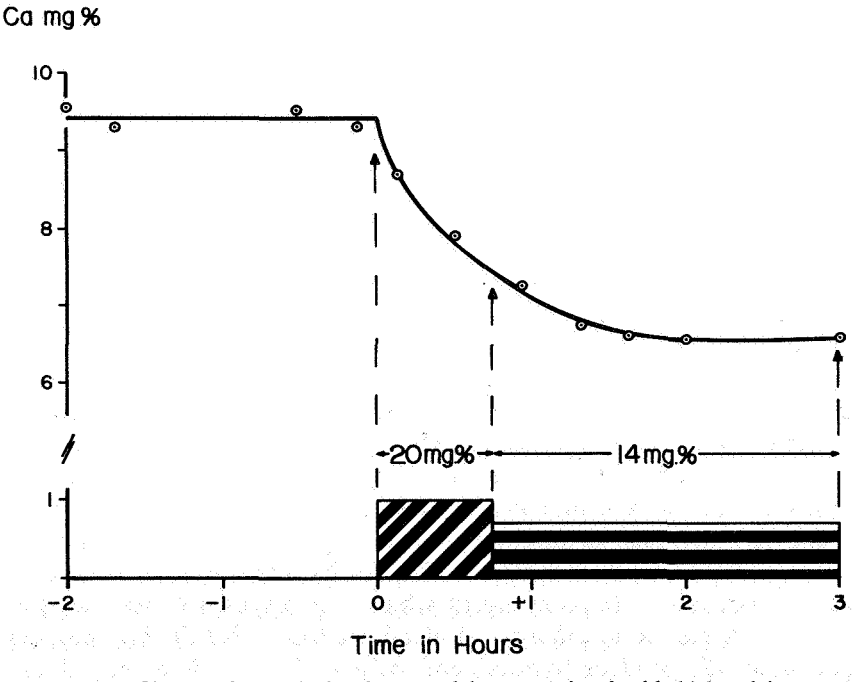

FIGURE 30. Changes in systemic plasma calcium associated with high calcium perfusion of the pig thyroid *in situ*. The calcium concentration in the perfusing blood is indicated. [From data in ref. 66; reprinted by permission of the author.]

COPP: Dr. Bélanger should comment on this point. All thyroid cells appeared larger, but the effect was most marked in the parafollicular cells. Dr. W. Chase prepared electron micrographs which showed increased activity in the endoplasmic reticulum of these cells.

BÉLANGER: Of course, these are old names which date back to the 1920's. It is now possible to demonstrate that these are two aspects of the same cell, and that the parafollicular cells, which are not parafollicular at all, are away from the follicle and are distal to the colloid. These are young cells that are possibly capable of responding to STH-stimulation but not to TSH, and are capable of manufacturing thyrocalcitonin but not thyroid hormone as we understand it.

Now, as these cells mature and move toward the colloid to replace the cells that are dying out there, they also acquire the ability to manufacture thyroid hormone. Therefore, in our estimation, all types of cells can make thyrocalcitonin, but only the more adult, or more mature, cells can make thyroid hormone.

PECK: Did you find any evidence for increased thyroxine genesis?

COPP: With calcium?

PECK: Yes.

COPP: No. That would be an interesting experiment but we have not done it yet.

BÉLANGER: There is histochemical evidence to the effect that the production of thyroid proteolytic enzyme, which is associated with the utilization of thyroglobulin, actually is stimulated by treatment such as Dr. Copp has described; that evidence would indicate that both functions are enhanced.

COPP: Both Dr. Bélanger and we have observed some thyroid hyperplasia in rats made hypercalcemic by feeding a high calcium, low phosphate diet. However, the effects were not as striking as in the sheep.

NICHOLS: Do you think the calcitonin is stored with the colloid, or is this very rapid change a secretory phenomenon?

HEANEY: Apparently there is a vast difference in the amount of colloid. It seems inconceivable to me that an equivalent amount of thyroid hormone could have been secreted under the conditions that would not have affected——

BELANGER: I do not know about the physiologic detection, but certainly since the proteolytic enzyme production is also increased, it seems that as the utilization of this thyroid hormone is increased, then the residual amount of colloid could very well not be representative of the amount of production of the hormone, because the turnover rate also appears to be considerably greater.

COPP: We should do this experiment sometime. It would be very interesting to see the effect on protein-bound iodine (PBI).

TALMAGE: Two years ago, when we made our first report concerning the presence of thyrocalcitonin in rats, we noted that the release of thyrocalcitonin which followed parathyroidectomy was also accompanied by a very marked increase in ^{131}I-PBI (ref. 67).

COPP: I would now like to turn the meeting over to Dr. Arnaud so that he can tell us of the very exciting work in his laboratory on the chemistry and secretion of thyrocalcitonin.

ARNAUD: Thank you, Dr. Copp. I should like to present the work which Drs. Tenenhouse, Rasmussen, and I have done on the isolation and characterization of porcine thyrocalcitonin (ref. 68) and also discuss some preliminary studies of a radioimmunologic assay system for the polypeptide that Dr. Littledyke and I have developed. All presently available evidence indicates that this polypeptide is the hormone thyrocalcitonin; but it is important to point out that Baghdiantz et al. (ref. 69) have reported a purified preparation of thyrocalcitonin that may have a much smaller molecular weight. Further studies are

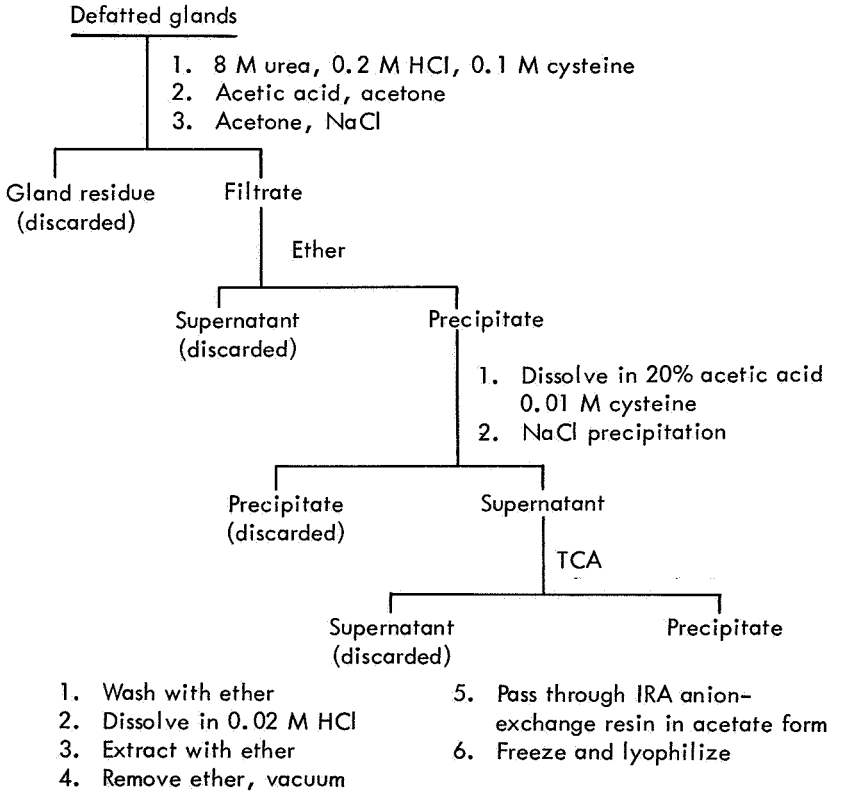

FIGURE 31. Flow diagram of the extraction of thyrocalcitonin from dried, defatted, fresh, frozen, porcine thyroid glands. Final product is a crude trichloroacetic acid (TCA) powder.

required before the possible relationships between these two products can be known.

As shown in figure 31, dried and defatted, fresh, frozen porcine thyroid glands are carried through urea-HCl-cysteine extraction and solvent, salt and trichloroacetic acid (TCA) precipitation procedures resulting in a crude powder extract. This procedure is almost identical with that applied to bovine parathyroid glands in our purification procedure for parathyroid hormone (ref. 70). This crude powder extract is relatively stable when refrigerated in the lyophilized state at 4° C and produces a decrease in the plasma calcium of calcium-deficient rats of 1.5 mg/100 ml at an intravenous dose of 30 to 40 micrograms of protein (ref. 71) 1.5 hours after administration. This crude powder is further purified by dextran gel chromatography on columns of Sephadex G–75 using 0.2 *M* ammonium acetate, pH 4.6 as eluant. The eluted material is pooled and lyophilized, as shown in figure 32. Although considerable biologic activity is observed in both pools, tubes 92 to 100 and tubes 100 to 120, the greatest was present in the former, and it was chosen for further characterization.

When this material is subjected to either starch gel (fig. 33) or polyacrylamide (fig. 34) electrophoresis, it migrates as a single component.

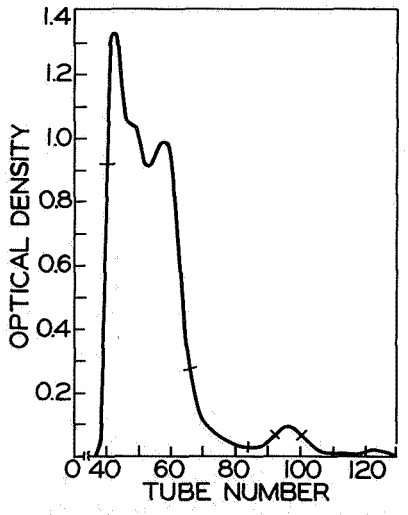

FIGURE 32. Gel filtration of crude thyroid extract (see fig. 31) on Sephadex G–75 column 2.5 times 130 centimeters. The eluant was 0.2 *M* ammonium acetate, pH 4.6. The peak in tubes 92 to 102 contained the major portion of the biologic activity. [From tenenhouse et al., ref. 68.]

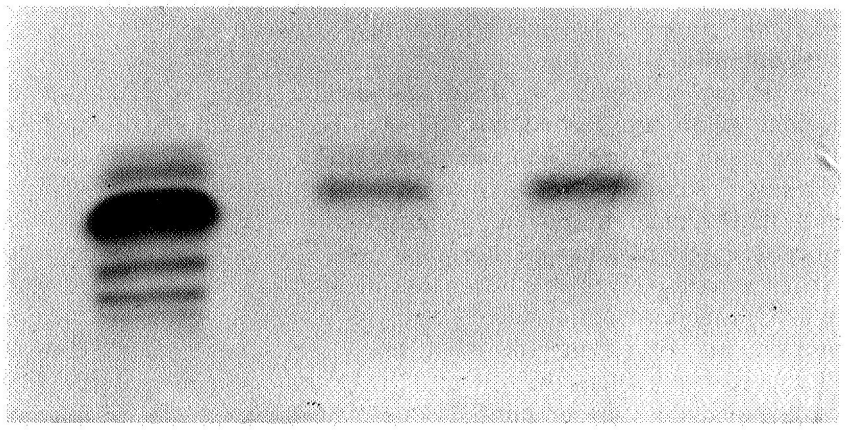

FIGURE 33. The patterns obtained on starch gel electrophoresis when crude and purified preparations of thyrocalcitonin were analyzed. Crude TCA powder (left), active fraction from gel filtration on G–100 (middle), and active fraction from gel filtration on Sephadex G–75 (right). The material on the right is the substance which was further characterized. [From Tenenhouse et al., ref. 68.]

It has a molecular weight of approximately 9000, and amino acid analyses (table I) show that it is unique in that it has a single, half-cystine residue.

HEANEY: Dr. Arnaud, in virtue of our past discussions, are there any iodinated amino acids in that molecule?

ARNAUD: I will tell you about an interesting finding. The $-SH$ group of the cystine residue is not titrable, so that it is not free, at least not by any criteria that we have been able to determine. It is possible that an iodine molecule might be linked with this $-SH$ group; we have not been able to look into this yet. But, it is important to remember one thing in this regard. The hormone-extraction procedure involves the addition of cysteine at two separate points; it is possible that the molecule might bind this amino acid so that the final product contains a cysteine residue in nonpeptide linkage.

We have been able to produce antibodies to this purified material by conjugating it with carbodiimide (ref. 72) to rabbit albumin and by injecting the conjugated material into rabbits with Freund's complete adjuvant. Figure 35 is a gel diffusion pattern in which we have placed the antibody in the center well (IV), a homogenate of porcine thyroid tissue in well III, the crude TCA extract in well I, and the purified material in well II. Therefore, the antibody is quite specific for the isolated peptide in that lines of identity are present.

Figure 36 is a microimmunoelectrophoretic study of the crude TCA extract (I) and purified preparation (II) with antibody in the longitudinal center well (IV). There is a single band of precipitation where

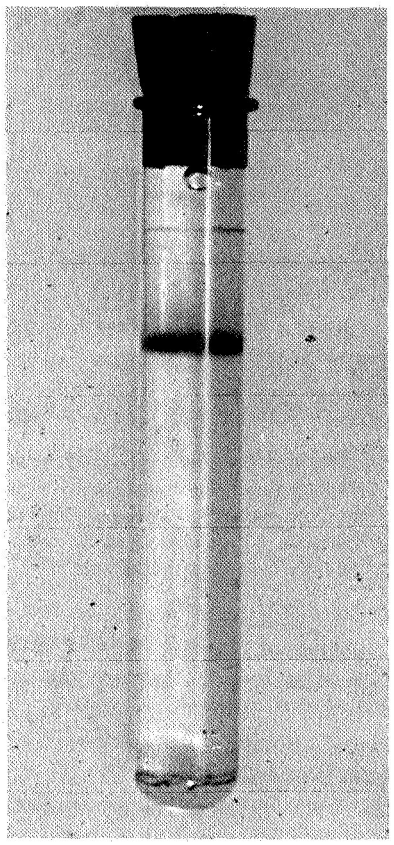

FIGURE 34. Pattern obtained on polyacrylamide disk electrophoresis of the active fraction from gel filtration on Sephadex G–75. Sample size was 100 micrograms.

antibody and antigen have reacted, which again supports the idea that the prepared antibody is specific for the isolated polypeptide.

We decided that we would like to find out which cells of the thyroid gland produce this polypeptide. With Gary Hargis and Gerald Williams in Chicago, we carried out a series of immunofluorescent studies of the thyroid gland with the antibody conjugated to the fluorescent material, lissamine rhodamine B 200 (ref. 73). We see (fig. 37) fluorescence over the cytoplasmic material of all the thyroid cells of the pig. The thyroid glands of the dog, rat, and human have been studied in a similar manner with similar results. Notice that there is no specific fluorescence over any of the colloid.

PECK: Can you block that fluorescence?

TABLE I

AMINO ACID COMPOSITION OF THYROCALCITONIN

Amino acid	Residues per molecule			
	22 hours	72 hours	22 hours oxidized	Calculated
Cysteic acid	—	—	1.07	1
Aspartic acid	7.06	6.86	—	7
Threonine	3.69	3.26	3.95	4
Serine	3.76	3.00	3.48	4
Glutamic acid	9.72	9.25	—	10
Proline	4.36	4.86	—	4
Glycine	5.22	5.06	5.42	5
Alanine	7.82	7.83	7.95	8
Valine	4.24	4.20	4.25	4
Cystine	.50	—	—	.50
Methionine	.90	.67	—	1
Isoleucine	2.79	2.76	2.84	3
Leucine	9.15	8.96	9.27	9
Tyrosine	2.22	2.00	—	2
Phenylalanine	2.13	2.20	1.99	2
NH_3	8.03	10.16	—	7
Lysine	8.38	8.29	8.08	8
Histidine	1.22	1.27	1.02	1
Arginine	3.60	3.48	3.62	4
Tryptophan	1.00	.....	—	1

ARNAUD: Yes. It is specifically blocked in two different ways. If we overlay the tissue with nonfluorescinated antibody, fluorescence is totally blocked. Similarly, incubation of the fluorescinated antibody with the isolated polypeptide overnight at 4° C before overlaying the tissue results in the complete absence of fluorescence.

We became intrigued with the idea that parathyroid hormone and thyrocalcitonin might be chemically similar because of their similar molecular weights. If one lines up the two molecules in terms of their amino acid residues (table II), they compare rather closely. There are some important differences, however. Of particular note is the presence of three histidine residues in parathyroid hormone, compared with only one in the thyrocalcitonin peptide, and the absence of cysteine in parathyroid hormone. Further, when one elutes these polypeptides from carboxymethyl cellulose (CMC) columns with identical concentration gradients of ammonium acetate (fig. 38), parathyroid hormone comes off at a concentration of 0.33 *M* and the thyrocalcitonin peptide at 0.1 *M*, indicating that parathyroid hormone is a more highly charged molecule.

311-496 O-68—6

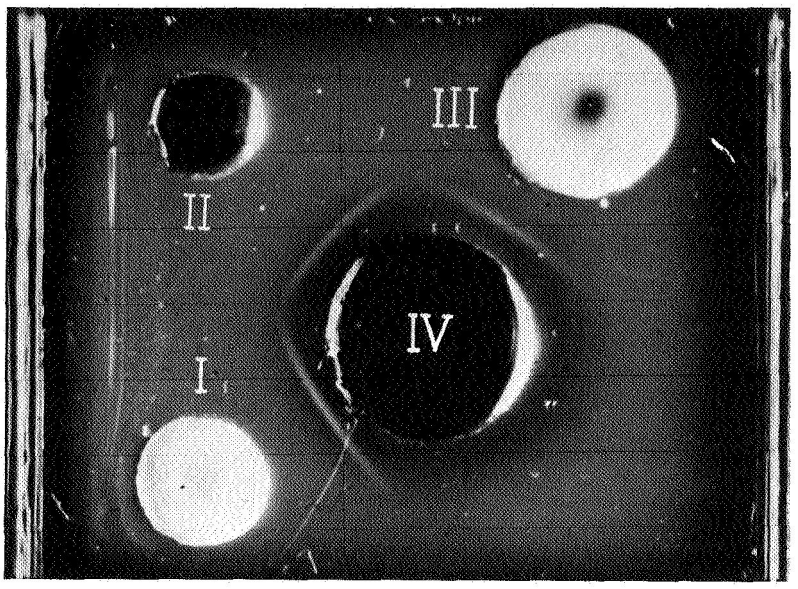

FIGURE 35. Microimmunodiffusion pattern of reaction between antiserum to TCT and three preparations of TCT, showing pattern of fusion. I, crude porcine TCT extract; II, active fraction from G–75 fractionation; III, homogenate of porcine thyroid tissue; IV, rabbit antiserum to material in II. [From Hargis et al., ref. 73; reprinted by permission of the publisher.]

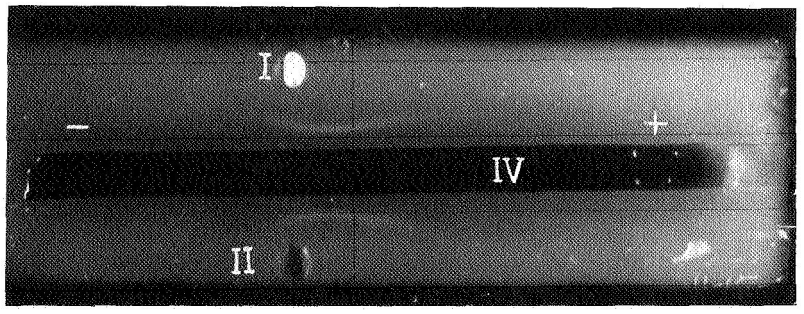

FIGURE 36. Microimmunoelectrophoresis pattern of reaction between antiserum to TCT and two preparations of TCT, showing single corresponding arcs in anodic position (barbital buffer, pH 8.8). I, crude TCA extract; II, active fraction from fractionation on G–75; IV, rabbit antiserum to II. [From Hargis et al., ref. 73; reprinted by permission of the publisher.]

Dr. Littledike, Miss Tsao, and I have developed a radioimmunologic procedure for the assay of this polypeptide in biologic fluids, which is similar to that described by Yalow and Berson (ref. 74) for insulin.

The radioimmunoassay depends on the competitive inhibition of stable thyrocalcitonin with ^{131}I-labeled thyrocalcitonin for antibody binding, and the degree to which stable thyrocalcitonin will inhibit

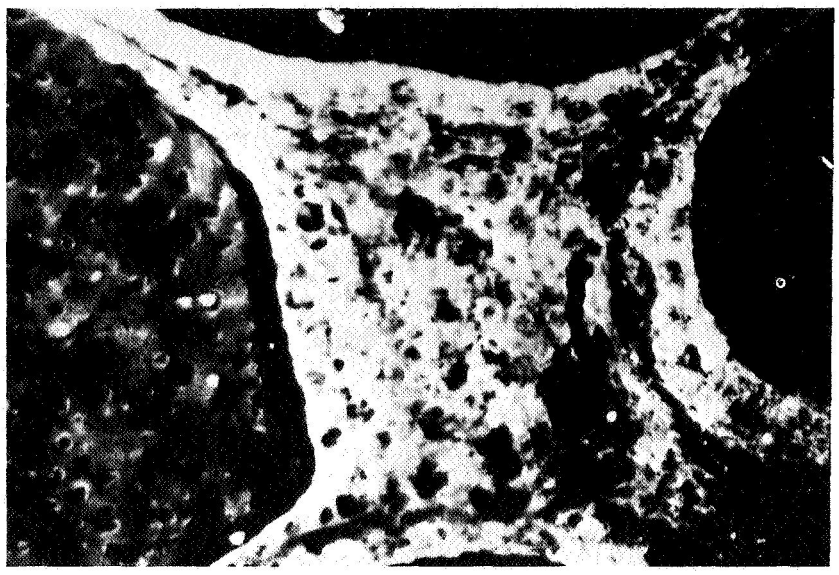

FIGURE 37. Porcine thyroid gland: fluorescent antibody globulin to TCT stain, showing fluorescence confined to thyroid epithelial cells. 400 ×. [From Hargis et al., ref. 73; reprinted by permission of the publisher.]

TABLE II

COMPARISON OF THYROCALCITONIN AND PARATHYROID HORMONE IN AMINO ACID COMPOSITION

	Thyrocalcitonin	Parathyroid hormone
Aspartic	7	8
Threonine	4	1
Serine	4	6
Glutamic acid	10	10
Proline	4	2
Glycine	5	4
Alanine	8	6
Valine	4	7
Cystine	.5	0
Methionine	1	2
Isoleucine	3	3
Leucine	9	7
Tyrosine	2	1
Phenylalanine	2	2
NH_3	7	7
Lysine	8	7
Histidine	1	3
Arginine	4	4
Tryptophan	1	1

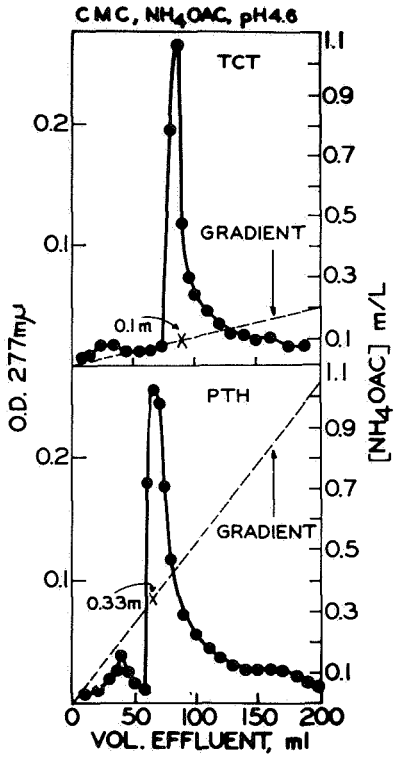

FIGURE 38. Column chromatography of porcine TCT polypeptide and purified parathyroid hormone (PTH) on carboxymethyl cellulose using increasing ionic strength gradient, ammonium acetate, pH 5.0. Major protein peak of PTH elutes at 0.33 *M* and that of TCT at 0.1 *M*.

iodinated thyrocalcitonin from being bound to antibodies is proportional to the concentration of stable thyrocalcitonin added to the mixtures.

Antibody-bound ^{131}I-labeled peptide is separated from free ^{131}I-labeled peptide by a chromatoelectrophoretic technique on Whatman 3 MC filter paper strips and subsequently evaluated by means of a chromatogram strip counter and peak area integration. Figure 39 shows a series of tracings obtained when progressively increasing quantities of stable peptide are added to mixtures containing the same amount of antiserum (1 to 50 000 dilution) and labeled peptide after 4 days' incubation at 4° C. As you can see, increasing the concentration of stable peptide is associated with a decrease in the quantity of labeled peptide bound to antibody.

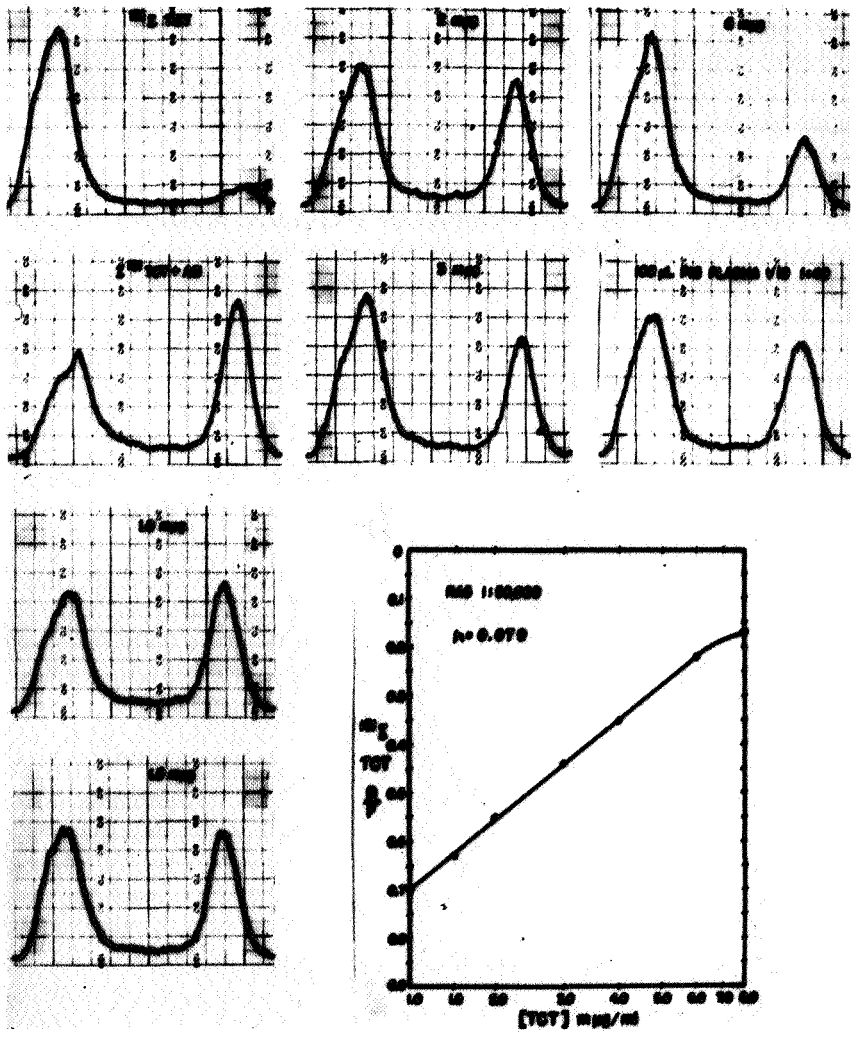

FIGURE 39. Scans of radioactivity on paper-strip chromatoelectrophoretograms of mixtures of ^{131}I-labeled porcine thyrocalcitonin (TCT) and rabbit antiporcine TCT antiserum (dilution 1 to 50 000) in the same concentration but with varying concentrations of unlabeled porcine TCT as indicated. The right of the two peaks on each tracing represents migrating antibody bound, and the one on the left, free ^{131}I-labeled peptide which remains at the origin. The tracing marked "^{131}I TCT" is a scan of a mixture to which no antiserum had been added and the small migrating peak "damaged" ^{131}I TCT. No unlabeled TCT was added to the mixture represented by the tracing marked "^{131}I TCT + AB." The standard curve (right) was obtained by measuring the areas under the bound and free peaks of each scan, expressing them as ratios (B/F) and plotting this value against the logs of the corresponding known concentrations of added, unlabeled TCT. The scan just above the standard curve was obtained when the peripheral plasma of a calcium loaded intact young pig was substituted in a dilution of 1 to 40 for unlabeled TCT in the mixture. Its concentration was calculated as 500 mμg/ml of whole plasma.

If one plots the ratios of the areas under these curves (the ratio of the antibody bound to free labeled peptide) against the log of the concentration of added stable peptide, one obtains a straight line. To measure the concentration of peptide in an unknown solution, one merely adds an aliquot to the mixture of antibody and labeled peptide in place of the standard stable peptide. The assay can easily detect 1.0 mμg of peptide per milliliter of incubation mixture. Greater sensitivity can be achieved but it is not necessary because the concentrations in normal plasma are high (young pig, 100 to 400 mμg/ml). In nine consecutive assays, the index of precision (λ) was 0.079.

The concentration of antibody is critical. If too much is added, the competition between labeled and stable peptide for antibody binding becomes difficult to demonstrate, and the sensitivity of the assay suffers.

Using this radioimmunoassay as a sensitive tool, we sought to determine if there was any immunologic similarity between highly purified bovine parathyroid hormone and the thyrocalcitonin molecule. To our surprise, minimal crossreactivity could be demonstrated.[1]

Our attempts at radioimmunoassay in the beginning were quite crude, and my attempts during the past 6 months have been primarily to improve the selection of antisera and to look at thyrocalcitonin from other species.

Since my background is internal medicine, I was interested to see if a polypeptide similar to the one we isolated from porcine thyroid glands was present in human plasma. Figure 40 is from a study made using serial dilutions of my plasma in the immunoassay system. The value for the bound-to-free ratio obtained with my undiluted plasma falls directly on the curve obtained with standard porcine peptide; with serial dilution, an almost identical set of points is observed. This indicates that an immunologically similar polypeptide circulates in human plasma, and it can be precisely measured in terms of porcine peptide equivalents.

We have not really gone very far in measuring the hormone in disease states. All we can say is that it circulates in substantial quantities. We have obtained from 25 normal sera, or relatively normal sera, an average of 30 to 50 mμg/ml. This is on the order of 50 times the level of parathyroid hormone which circulates in bovine plasma (ref. 40).

URIST: Have you seen a patient with idiopathic hypoparathyroidism?

ARNAUD: I have measured the polypeptide in the plasma of these individuals, and I do not think it is increased; but I would not like to go on record.

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[1] Exhaustive studies now indicate that the presence of crossreactivity was a result of glassware contamination.

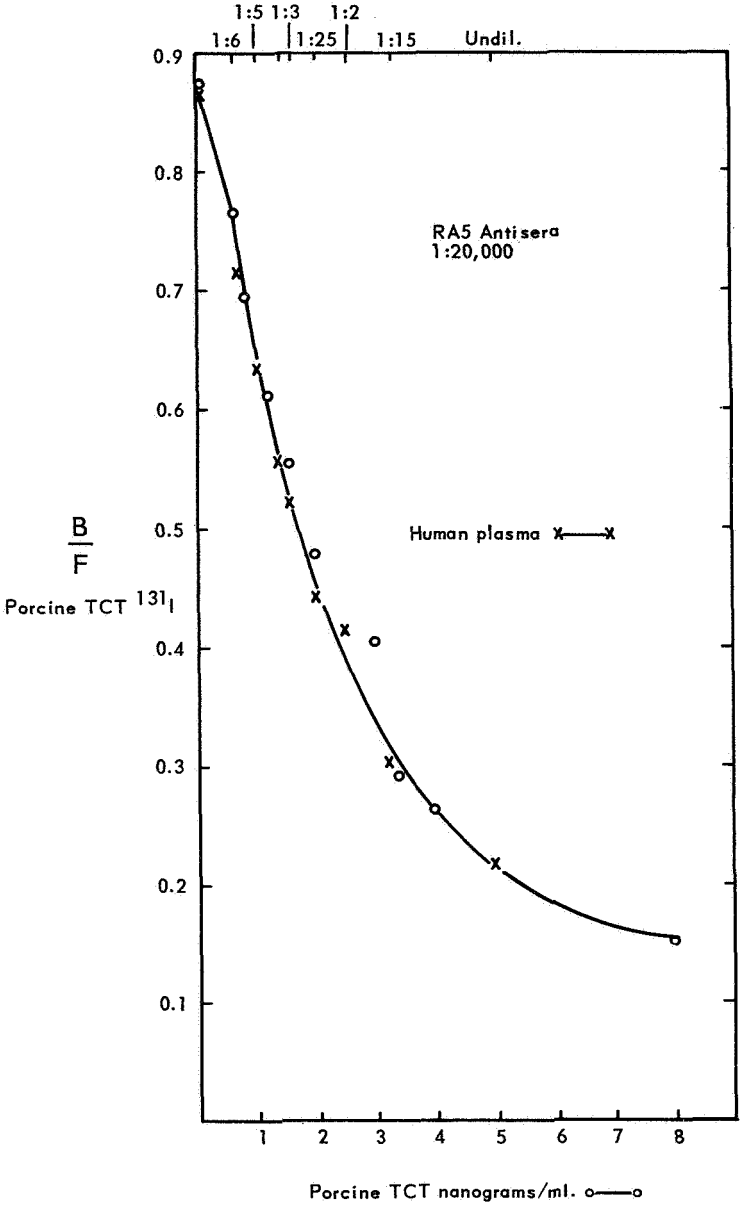

FIGURE 40. Plot of the ratio of antibody bound to free ^{131}I porcine TCT peptide against the concentration of added stable porcine TCT peptide (×———×) and human plasma in various dilutions (○———○). Similarity of curves indicates that a substance which circulates in human plasma is immunologically similar to the added porcine TCT peptide.

WALSER: Have you done a thyroidectomized subject?

ARNAUD: Only thyroidectomized pigs. It is much lower, but quite consistently present in all of the animals thus far studied. It is possible that this is due to ancillary thyroid tissue, but this finding concerns us very much and has led us to consider alternative possibilities, such as, that the polypeptide that we isolated may be produced by tissues other than the thyroid gland or that there may be a carrier of the hormone (thyrocalcitonin).

COPP: Have you measured this in osteoporotic patients?

ARNAUD: No; we have not. I have been able to measure thyrocalcitonin in human plasma for 4 weeks now.

NICHOLS: I would like to know where thyrocalcitonin works in the bone.

RAISZ: According to figure 22, thyrocalcitonin acts to inhibit bone resorption. There is some preliminary evidence that this is the way thyrocalcitonin works *in vivo*. I think Dr. Talmage has evidence on this point. The evidence for inhibition of bone resorption in tissue culture is fairly clear. We measure the release of ^{45}Ca from prelabeled bone in tissue culture into a medium which contains a large amount of stable calcium, so that major changes in this release can be achieved only by affecting resorption. Changes in deposition of calcium would take calcium largely from the unlabeled pool and very little ^{45}Ca would be returned to the bone. Our experiments (ref. 75) show that in tissue cultures with and without parathyroid hormone, the release of radiocalcium is largely inhibited by the action of thyrocalcitonin (table III).

TABLE III

EFFECTS OF A CRUDE THYROCALCITONIN PREPARATION (TC–J) AND PARATHYROID HORMONE (PTH) ON ^{45}Ca RELEASE FROM EMBRYONIC BONES [a]

	Dose		
	3 μg/ml	10 μg/ml	30 μg/ml
TC–J	910	700	770
Control	920	920	1050
Difference	10 ± 64	[a] 220 ± 55	[a] 280 ± 80
TC–J + PTH	1510	780	890
PTH	1880	1580	1750
Difference	370 ± 180	[a] 800 ± 60	[a] 860 ± 180

[a] Difference significant by t test, $p < 0.02$. Values, expressed as cpm/0.1 ml medium, are means for ^{45}Ca release for 3 or 4 bones during 48-hour culture, and mean ± SE for the difference between paired bones. [Adapted from Friedman and Raisz, ref. 75.]

Since this experiment was done we have more refined materials, and a partially purified rat thyrocalcitonin has been prepared which is effective in doses of as little as 0.1 μg/ml. When parathyroid hormone is added to the culture, calcium release is increased in bones that are not treated with thyrocalcitonin; but the entire parathyroid effect is brought back to nonresorbing levels by thyrocalcitonin. In addition, thyrocalcitonin inhibits bone resorption in the absence of parathyroid hormone; i.e., what we call control bone resorption.

BÉLANGER: There are some other experiments that have a bearing on this matter. These are the experiments, done immediately before this conference, in which we have evidence that the thyroid gland has been stimulated into taking up the kind of aspect that Dr. Copp has shown. We did this in rats, and then looked at the bones and observed a thyrocalcitonin effect; but it is some sort of an effect that is inhibitory under, let us say, thyrocalcitonin production-like conditions.

There are areas of the bone where the cells become enlarged and are actually surrounded by areas of matrix in which the staining properties will change; for example, under these conditions the usual color with the use of Wright's stain will change to an azure staining.

Now, by comparing this type of staining with a known stain for metachromasia, such as methylene blue, the same areas in the vicinity of the large cell show some metachromasia. This is generally considered as indicative of the presence of some mucopolysaccharides.

In the tibias from rats on a diet low in phosphate, we observed a complete disappearance of these zones of metachromasia except where there was a remnant of cartilage. There are no more of these hypertrophic osteocytes; the osteocytes now appear to be small, and some of them have even died. If you look in other areas more reactive than the tibia, such as the alveolar bone between the molars, we can see that some of these cells, after 7 days of this type of treatment, become totally degenerated. I suggest at this point that if this is indicative of a thyrocalcitonin effect, then the thyrocalcitonin effect is manifested by an inhibition of the metabolism of the large osteocytes which preside over osteolysis.

URIST: What is the target of thyrocalcitonin? Dr. Bélanger, do you suggest that calcitonin controls the process of osteolysis?

BÉLANGER: No, not quite. Osteolysis is the end product, but I think there is some sort of change in the metabolism of the osteocyte whereby it cannot perform its normal osteolytic role—particularly in the large, mature osteocyte which is actually responsible for osteolysis. The osteolytic role may be due to the production of some acids, as Dr. Nichols has mentioned, or perhaps due to the production of some enzymes that break down the organic matrix of the bone. These

effects, then, do not take place, and the cells appear either to remain small or become small. I do not know at the moment.

URIST: Is the osteocyte a target?

BÉLANGER: Yes. This is what these experiments seem to indicate.

URIST: Do you agree with Dr. Raisz, that the osteoclastic activity is inhibited by thyrocalcitonin?

TALMAGE: He did not say. He just said bone resorption. I do not think that we know which cell is responsible.

RAISZ: I only said bone resorption. The response that I showed earlier to parathyroid hormone in the first 3 hours, during which no cellular changes can be seen, is also inhibited by thyrocalcitonin.

BÉLANGER: Osteoclasts are nowhere around.

TALMAGE: I think it is important to emphasize that thyrocalcitonin is just as active in parathyroidectomized animals as it is in control animals. In our experimental system, we place the animal under a calcium stress by our peritoneal lavage system. By this method calcium is removed from the animal at a maximum rate; in the animals maintained on a calcium-free diet, this calcium must be supplied by bone. By such a system, we are magnifying the progress of bone resorption and minimizing bone accretion. If the animals are given thyrocalcitonin during this process, the rate of calcium removal is diminished equally in both control and parathyroidectomized animals. The only difference is the length of time in which the effect lasts. In the animal with intact parathyroids, it reaches its peak effect in 1 hour and returns to normal in 2 or 3 hours. Without parathyroids, it reaches its peak in 2 or 3 hours and may not return to normal for 5 or 6 hours.

The point should be made that phosphate drops proportionately with calcium. Another interesting point pertains to the removal of radioisotopes of calcium and phosphate administered 18 hours or 3 weeks prior to treatment. Thyrocalcitonin caused a reduction in the removal rate during peritoneal lavage with a calcium-free rinse. This reduction was seen in all animals. In parathyroidectomized animals, only long-term administered radioactivity is affected. Because of this difference we do not feel that the two hormones, while producing opposite effects, necessarily work at the same loci in bone cells.

COPP: I might point out that both Dr. Munson and Dr. Wase have some indirect evidence that thyrocalcitonin may also enhance the deposition of calcium in bone.

BÉLANGER: That is secondary.

PRITCHARD: I must comment for a moment on the histologic problem of interpreting osteocyte behavior. It seems very odd that the osteocytes farthest from the blood vessels should be the ones to react. I am not happy about the interpretation given. I think the explanation

is that the osteocytes of primary woven bone are different from the osteocytes of secondary lamellar bone.

COPP: I would like Dr. Arnaud to show his experiment demonstrating the control of thyrocalcitonin secretion.

ARNAUD: This is an experiment done in a female pig weighing 55 kilograms (fig. 41). We measured plasma calcium, phosphate, and immunoassayable thyrocalcitonin peptide before and after calcium loading, when the animal was intact and after its thyroid gland had been surgically excised. The maximum for thyrocalcitonin is about 160 mμg/ml.

This study was made at a time when the radioimmunoassay was relatively insensitive and normal plasma levels could not be measured. When a calcium load was given to the animal when its thyroid was intact, a modest degree of hypercalcemia was observed. Coincident with the hypercalcemia, there was a precipitous rise in the level of

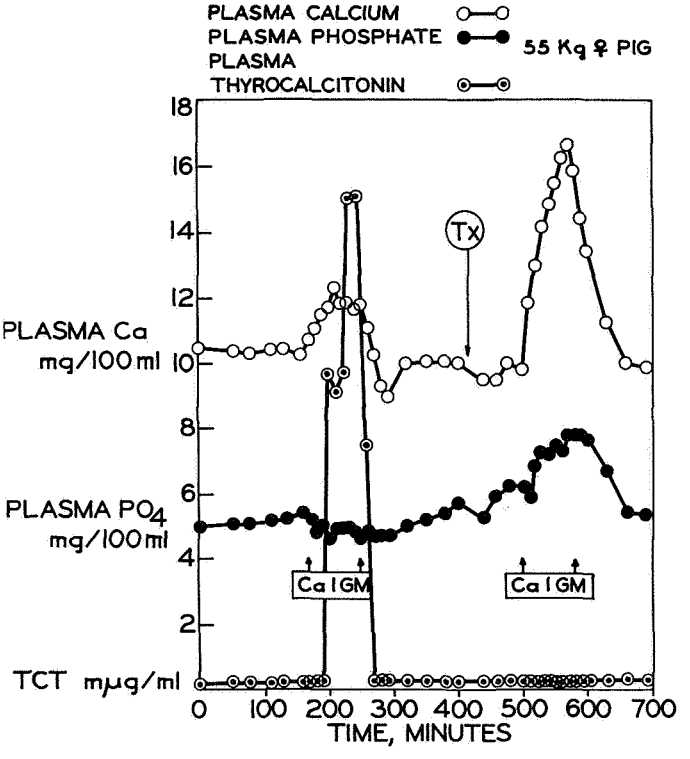

FIGURE 41. Effect of intravenous calcium loading on the concentration of calcium, phosphate, and thyrocalcitonin (TCT) in the arterial plasma of a pig before and after thyroidectomy. TCT levels below 60 mμg not measurable at this time in the development of the radioimmunoassay. (Multiply scale for TCT times 10.)

thyrocalcitonin in the plasma. After the calcium loading was discontinued, you can see that there is a relatively rapid return of the plasma calcium to control, perhaps to below control levels, and an immediate decrease in the concentration of thyrocalcitonin to nonmeasurable levels. This occurred over a period of 20 minutes or so; it is a rough estimate of the rate of disappearance of endogenously secreted polypeptide.

In contrast, a markedly different calcium curve is observed when a calcium load is given to the same animal after thyroidectomy. The degree of hypercalcemia that develops is two to three times greater than when the thyroid gland is present, while the level of thyrocalcitonin remains undetectable.

COPP: I would like to thank all of the participants in this session.